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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a Provisional Application under 37 C.F.R. 1.53(c).

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2. **TITLE: Activation of Hypoxia-inducible Gene Expression**

3. **APPLICATION PAPERS ENCLOSED**
   29 Pages of Specification
   21 Sheets of Drawings

4. **METHOD OF PAYMENT - FILING FEE - $80.00 (Small Entity)**
   The Commissioner is hereby authorized to charge **$80.00** to Deposit Account **50-0310** for payment of the provisional application filing fee. Applicants qualify as a small business concern as defined in 37 C.F.R. 1.27(a)(1) through (a)(3) for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code.

   Except for issue fees payable under 37 C.F.R. 1.18, the Commissioner is hereby authorized by this paper to charge any additional fees during the entire pendency of this application including fees due under 37 C.F.R. 1.16 and 1.17 which may be required, including any required extension of time fees, or credit any overpayment to Deposit Account 50-0310. This paragraph is intended to be an **constructive petition for extension of time** in accordance with 37 C.F.R. 1.136(a)(3).

5. **GOVERNMENT CONTRACT**
   The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government. The name of the U.S. Government Agency and the Contract Number are: NIH Grant NS37814 and DOD Grant MDA 905-92-Z-0003.

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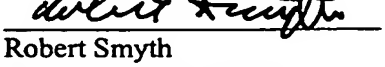

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Activation of Hypoxia-Inducible Gene Expression

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Field Of The Invention

The invention relates generally to the changes in gene expression in human tissues, which bring about improved survival in conditions of reduced blood flow and oxygen supply. The invention relates specifically to the pharmacological activation of hypoxia-inducible gene expression by 2-oxoacids and their derivatives.

Background Of The Invention

The ability to adapt to low oxygen levels, perhaps best known in the context of acclimation to high altitudes, is crucial for survival. Cells adapt to low oxygen by turning on genes that encode for proteins which promote better oxygen delivery via new red blood cell synthesis (erythropoiesis) and development of new blood vessels (angiogenesis). Other hypoxia-stimulated gene products stimulate glucose uptake, enhance anaerobic glucose metabolism, and induce several cell survival mechanisms (table 1). Athletes have long capitalized on such hypoxic adaptations to improve their physiological performance. In addition, the deliberate adaptation of cells to sublethal hypoxia has also been shown to reduce tissue injury from strokes and heart attacks. The hypoxic challenge in these settings, referred to as hypoxic preconditioning, has been shown in many animal studies to constitute one of the most potent strategies in reducing ischemic injury. Hypoxic preconditioning mediated protection against ischemic injury has been shown to occur *in vivo* in a variety of organ systems, including the heart, brain, spinal cord, retina, liver, lung and skeletal muscle (1). Ishchemic or hypoxic preconditioning is also useful in prolonging the survival and grafting efficiency of donated tissue used for transplants.

The mechanisms by which hypoxia induces the expression of survival promoting genes are rapidly becoming clarified. Hypoxia (oxygen levels below 5%) regulates gene expression predominantly via the transcription factor HIF-1 (hypoxia-inducible factor-1) (2). Two different proteins called HIF-1 alpha (HIF-1a) and HIF-1 beta (HIF-1b) make up this transcription factor and the level of the HIF-1a component is specifically regulated by oxygen tensions. The regulation of HIF-1a levels involves a novel oxygen sensing mechanism which directly controls the degradation of the HIF-1a protein (figure 1). Both HIF-1a and HIF-beta are constitutively synthesized in most cells of the body. However the HIF-1a protein is continuously degraded in the presence of oxygen. A newly discovered family of enzymes known as HIF-1-alpha-prolyl hydroxylases regulate the oxygen-dependent degradation of HIF-1a. These enzymes catalyze the oxygen-dependent hydroxylation of a key proline residue in the HIF-1a protein. This modification, in turn, directs the ubiquitination and proteasomal degradation of the HIF-1 protein. Another recently identified HIF-1a asparagine hydroxylase enzymatic activity also appears to be involved in inhibiting the trascriptional activation ability of HIF-1 under normal oxygen tensions. The HIF-1a asparagine hydroxylases has been termed Factor Inhibiting HIF or FIH-1 by other investigators. All of the HPH and FIH-1 require several co-factors for their activity: oxygen, iron, ascorbate, and 2-oxoglutarate (figure 2). In the absence of oxygen therefore, HIF-1a is not hydroxylated or degraded, and as a result, its concentration increases dramatically (2). This allows the HIF-1a and beta subunits to dimerize, translocate to the nucleus and activate the transcription of several genes that promote survival under low oxygen levels (figure 1). The discovery of the HPH enzyme mechanism also explains why iron chelators such as desferrioxamine (DFO) can activate HIF-1 and turn on genes similar to those induced by hypoxia. Cobalt and nickel salts, which presumably compete for the iron sites in HPH also mimic hypoxia in regulating HIF-1 and hypoxic gene expression. Both DFO and cobalt have been used effectively to perform hypoxic preconditioning mediated cell protection in animal models of disease (3). Although the toxicity of these agents precludes their use in humans, their ability to induce protective preconditioning similar to that seen with hypoxia demonstrates that the pharmacological manipulation of HIF-1a levels by means other than hypoxia is a powerful therapeutic strategy. Recently, molecular interactions at the other cofactor sites have also been shown to regulate HPH activity, HIF-1a levels, and the expression of hypoxia-inducible genes. Thus, artificial analogs of 2-oxoglutarate, such as N-oxalylglycine (NOG) or the cell permeant dimethyloxalylglycine (DMOG), have been shown

to block the activity of the HPHs and FIH-1 and thus allow activation of HIF mediated gene expression (4). However, these artificial 2-oxoglutarate analogs are not specific in inhibiting HPHs or FIH-1 as they were initially designed to inhibit procollagen proline hydroxylases, the enzymes involved in collagen synthesis.

The HPH, FIH-1, and procollagen proline hydroxylases all belong to the large class of enzymes know as iron and 2-oxoglutarate dependent dioxygenases. These enzymes occur widely in nature and perform valuable biological hydroxylations (5). The reaction cycle for these enzymes is depicted in figure 2. One peculiarity of these enzymes is that they are syn-catalytically inactivated. This means that as a result of catalyzing iron mediated oxidations, these enzymes either become oxidized at critical amino acid residues or the redox state of the iron becomes useless in carrying out sustained reaction cycles. This syn-catalytic inactivation can be prevented and or reversed by ascorbate (figure 2). Many cell lines have recently been shown to express significant HIF-1a protein levels and HIF-mediated gene expression in the absence of hypoxia and this is reversible by ascorbate (6). This suggests that, in many cells, HPHs and FIH-1 may exist in an inactivated form or may be made inactive by some mechanism that is ascorbate reversible. So far, no clear understanding of this phenomenon has been achieved and no pharmaceutical approach has been developed to take advantage of a potential HPH and FIH-1 inactivating mechanism.

Certain pharmacological agents such as iron chelators, iron displacing metals, or 2-oxoglutarate antagonists such as NOG or DMOG are general inhibitors of the 2-oxoglutarate dependent enzymes. This family of enzymes is also differentially sensitive to a variety of naturally occuring 2-oxoacids and their derivatives (5,7,8). Thus, pyruvate does not inhibit the collagen synthesizing enzymes in humans (9) but does inhibit such enzymes in certain underwater dwelling worms (8). Although 2-oxoglutarate derived inhibitors that were developed for the inhibition of collagen synthesis do inhibit HPHs and FIH-1, the specific chemical requirements for 2-oxoacid molecules that inhibit HPHs and FIH-1 have not yet been elucidated. Glucose metabolism generates 2-oxoacids, such as pyruvate and oxaloacetate, that are structurally related to 2-oxoglutarate (figure 3). Amino acid metabolism also generates branched chain 2-oxoacids structurally resembling 2-oxoglutarate. It is possible that these naturally occuring 2-oxoacids are biological regulators of HPHs and FIH-1. It is also possible that these agents and their derivatives may be used to develop novel pahrmaceutical agents to regulate hypoxic gene expression.

Summary of the Invention

The present invention relates to the elucidation of specific molecular features of endogenous 2-oxoacid molecules and their derivatives for activating hypoxia-inducible gene expression by inactivating hypoxia-inducible factor hydroxylating enzymes. This invention identifies agents that can be used to induce tissue vascularization, treat anemias, induce tolerance to stroke and heart attacks, improve tissue healing and improve organ transplantation.

Brief Description Of Figures

Figure 1: HIF-1a hydroxylases and the regulation of gene expression by hypoxia

(A) HIF-1a protein hydroxylases are the best candidates for oxygen sensors in multicellular organisms to date. These enzymes require 2-oxoglutarate, ascorbate, Oxygen, and iron, thus explaining their inhibition under hypoxia or by iron chelators such as desferrioxamine (DFO) and competing metals such as cobalt. It is not known whether molecular interactions at the other indicated cofactor sites can regulate the activities of these enzymes. (B) Regulation of gene expression by hypoxia via HIF-1a protein hydroxylase activity. Two separate activities hydroxylate HIF-1a on distinct proline and asparagine residues to regulate the proteolysis and transactivating activity of HIF-1 respectively. These activities are inhibited under hypoxia allowing HIF-1a to accumulate and for the HIF-1 complex to activate gene expression (dashed lines). Abbreviations: DFO = desferrioximine, 2-OG = 2-oxoglutarate, Asc = ascorbate; bHLH = beta-helix-loop-helix domain, PAS = Per-Arnt-Sim domain, C-TAD = c-terminal transactivation domain, ODD = oxygen-dependent degradation domain, pVHL = von Hippel-Lindau protein, HIF-b = beta subunit of HIF, HRE = HIF regulatory element.

Figure 2: Putative enzymatic cycle for HIF prolyl hydroxylases

HPH and FIH-1 are members of the 2-oxoglutarate dependent dioxygenase enzyme family. These enzymes require iron, 2-oxoglutarate, and oxygen to carry out biological hydroxylations. This figure depicts a putative sequence of events that has been proposed for many members of this enzyme family (5). (A) HPH (grey C-shaped structure) bind iron (Fe). (B) The HPH-iron complex binds 2-oxoglutarate. The 2-oxo group coordinates with iron while the 5-carbon end of the molecule interacts with a different site. (C) This complex allows one atom of molecular oxygen to be inserted into the 2-oxoglutarte molecule to yield succinate and carbon dioxide while the other oxygen atom forms a complex with the enzyme-bound iron. (D) The iron-complexed oxygen is used to hydroxylate proline 564 within the HIF-1a oxygen dependent

degradation domain. HPHs also carry out similar hydroxylation on proline 402 while the FIH-1 enzyme hydroxylates asparagine 803. (E) Most enzymes that utilize this mechanism of hydroxylation become syncatalytically inactivated over time. This inactivation may involve redox reactions between oxygen, iron, and the enzyme and can be favored by certain conditions such as the presence of a pseudo-substrate. (F) Enzymes inactivated in this way can be re-activated with ascorbate which appears to bind these enzymes in a manner similar to 2-oxoglutarate.

Figure 3: Glucose metabolism and HIF-1 regulation

(A) Abbreviated scheme of glycolysis and strategy for determining the key glucose metabolite responsible for HIF-1a upregulation. During glycolysis, glucose is sequentially metabolized to pyruvate, which can then enter mitochondria for further metabolism or can be converted into lactate. Complex interconversions also link pyruvate and oxaloacetate (OAA) levels. The glucose analog 2-deoxyglucose (2DG) can only proceed to 2-deoxyglucose 6 phosphate and cannot be further metabolized. Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) is a key enzyme in glycolysis that can be selectively inhibited by iodoacetic acid (IAA). The transport of pyruvate and lactate across cellular membranes occurs through a specific carrier that is blocked by 4-hydroxycinnamate (4-CIN). Thus 4-CIN prevents pyruvate entry into mitochondria. The interconversion of lactate to pyruvate is mediated via lactate dehydrogenase (LDH), which can be selectively blocked by oxamate. Use of the various inhibitors and intermediates shown here allowed us to determine which key metabolite was responsible for HIF-1a activation. (B) Structural comparisons of 2-oxoglutarate (2-OG), succinate (Succ), oxaloacetate (OAA), and pyruvate (Pyr). 2-OG, OAA and Pyr are all 2-oxoacids based on the keto group at position 2, while succinate is not.

Figure 4: Regulation of HIF-1a levels by glucose metabolism

Western blots are shown in which nuclear extracts of U87 glioma cells were probed with a specific monoclonal antibody recognizing the HIF-1a protein. (A) U87 glioma cells cultured in DMEM were switched to Krebs buffer containing 5.5 mM glucose (Glc) and then evaluated for nuclear HIF-1a levels at various times by western blot analysis. (B) HIF-1a levels were measured after four hours incubation of cells in Krebs buffer containing the indicated glucose concentrations or with 5.5 mM 2-deoxyglucose (2-DG) substituted for glucose. (C) HIF-1a levels were measured in U87 cells cultured in glucose-free Krebs buffer following treatment for four hours under normoxia (21% oxygen) hypoxia (1% oxygen) or with 150 μM

desferrioxamine (DFO). (D) Induction of HIF-1a by glucose was monitored in the presence of 50 μM IAA or 1 mM 4-CIN. (E) HIF-1a levels were measured in U87 cells cultured for four hours in Krebs in which glucose was replaced with 3mM concentrations of lactate (Lac), pyruvate (Pyr), citrate (Cit), 2-oxoglutarate (2-OG), succinate (Succ), or alanine (Ala). Results are representative of experiments repeated at least three times. This figure demonstrates that HIF-1ą levels can be regulated byglycolytic metabolites and that the mechanism involved is non-obvious and distinct from that involving hypoxia.

Figure 5: Regulation of HIF-1a protein levels by lactate and pyruvate

U87 cells were maintained in MEM overnight. (A) The production of lactate in the culture buffer was measured over time following change from MEM to 5.5 mM glucose-containing Krebs buffer. Similar measurements were made in the presence of 50 μM IAA or in glucose-free Krebs buffer. (B) Buffer lactate and pyruvate levels were measured following four hour culture in 5.5 mM glucose containing Krebs buffer alone (open bars) or in the presence of 10 mM oxamate (closed bars) (C) Nuclear HIF-1a protein levels were determined four hours following switching of cells from MEM (control, CT) to Krebs containing either 0.55 mM glucose, or glucose replaced with the indicated concentrations of lactate or pyruvate. (D) HIF-1ą levels were measured after switching cells fromMEM to glucose-free Krebs containing 2 mM lactate or pyruvate. HIF-1ą induction by four hours treatment with 5.5mM glucose-containing Krebs (Glc) is shown for comparison. (E) Digitonin-permeabilized cells were treated with 1% oxygen or Krebs containing either 5.5 mM glucose or 2 mM pyruvate in the presence of 50 μM IAA. Permeabilized cell in lanes 5 and 6 were treated with 3 mM NAD or NADH respectively in glucose free Krebs. Nuclear HIF-1ąlevels were determined four hours later. (F) HIF-1a levels were determined after four hour treatment of cells in glucose–free Krebs (lane 1) or 5.5 mM Glucose containing Krebs. Glucose induced HIF-1a in both permeabilized or intact cells and neither NAD or NADH (3mM each) had any effect on this induction. Catalase (1000 and 2000units/ml) also had no effect. (G) HIF-1a levels were determined after four hours treatment in glucose-free Krebs containing 2 mM lactate or pyruvate with or without 10mM oxamate. (H) To measure the decay of the HIF-1ą protein, HIF-1a measurements were made after four hour treatment under hypoxia (lane 1), four hours hypoxia followed by 30 minutes normoxia (lane 2), four hour treatment with 150 μM DFO (lane 3), four hours DFO followed by addition of 100 μM CHX for one hour (lane 4), four hours treatment with 2 mM pyruvate (lane 5), and four hours pyruvate followed by addition of

100 μM CHX for one hour (lane 6). (H) HIF-1a levels were determined in digitonin-permeabilized cells treated for four hour with Krebs containing no glucose (lane 1), 1mM pyruvate (lane 2), or 1 mM pyruvate and 10 mM 2-OG (lane 3). Except where indicated, all experiments were carried out under normoxia and were repeated at least three times with similar results. This figure along with figure 3 demonstrates that of all the key changes that take place during glucose metabolism, it is the accumulation of pyruvate that promotes HIF-1a accumulation. Furthermore, pyruvate appears to mediating its actions by stabilizing HIF-1a protein levels.

Figure 6: Pyruvate analogs and oxaloacetate efficiently enhance HIF-1a protein levels Human U87 glioma cells (panels A-C) and other cell lines (D) were treated with glycolytic and Krebs cycle intermediates as well as ethyl and methyl esters of pyruvate and analyzed for HIF-1a accumulation in nuclear extract. With the exception of lactate, as discussed above, no other glycolytic intermediates were found to activate HIF-1a accumulation besides pyruvate. Of all Krebs cycle, only oxaloacetate (OAA) was able to stimulate HIF-1a accumulation. (B) The effects of pyruvate and OAA were mimicked by ethyl- and methyl-esters of pyruvate and were as pronounced as those of the known 2-oxoglutarate antagonist dimethyloxalylglycine (DMOG). (C) The effects of OAA were as potent as pyruvate and (D) were seen in several other cell lines including U251 human glioma cells, Hep3B human hepatoma cells, and DU145 human prostate carcinoma cells. Hela human cervical carcinoma cells, normal human astrocytes, and normal human prostate epithelium cells also displayed similar responses to pyruvate and oxaloacetate (data not shown).

Figure 7: Structure-activity requirements for 2-oxoacids that elevate HIF-1a levels HIF-1a protein levels accumulate due to inhibition of HPH activity as a result of either hypoxia, iron removal, or competitive antagonism of 2-oxoglutarate by artificial analogs such as N-oxalylglycine or dimethyloxalylglycine. We have found that naturally occurring 2-oxoacids can promote HIF-1a accumulation and their structural requirements for this activity are shown in this diagram. We determined the ability of each shown structure to induce HIF by exposing digitonin-permeabilized human glioma cells (U87, U251) to 1mM doses. N-oxalylglycine and its esterified precursor dimethyloxalylglycine consistently enhanced HIF-1a levels. Of all the other compounds shown, pyruvate, oxaloacetate, alpha-ketoisovalerate, alpha-ketoisocaproate, and alpha-keto-beta-methylvalerate (boxed) were the only agents capable of

stimulating HIF-1a accumulation. Lactate can also stimulate HIF-1a after its conversion to pyruvate (see figure 5). These data establish the necessity of the alpha-keto group. However, the ineffectiveness of alpha-ketobutyrate and alpha-ketoadipate also provide empirical data that other structural features are important.

Figure 8: HIF activation by 2-oxoacids is independent from energy metabolism

(A) U251 glioma cells were cultured in Krebs buffer without glucose or with the indicated additions at 2 mM each. At four hours, ATP levels were measured in cell extracts using the luciferase method. Although small variations in ATP were observed with the various treatments, there was no correlation with respective actions of these agents on HIF-1a accumulation. (B) Direct addition of 1 mM ATP to digitonin permeabilized cells also had no effect on HIF-1a levels.

Figure 9: Pyruvate stabilizes HIF-1a by acting at a step prior to ubiqitinylation

(A) U87 glioma cells were cultured for four hours in glucose free Krebs buffer alone (lane 1) or with the indicated treatments (DFO = 100 μM, DMOG = 1 mM, Glucose = 2 mM, Pyruvate = 2 mM, Lactacystin-beta lactone (Lbl) = 20 μM). All treatments except the 1% oxygen were performed under 20% oxygen. Whole cell extracts were then prepared and probed for HIF-1a protein levels. Only cells treated with the proteasome inhibitor Lbl, displayed HIF immunoreactivity with the characteristic larger molecular weight smear of ubiquitinylated HIF-1a. (B) U373 glioma cells were treated under conditions similar to those in (A). Oxaloacetate and succinate were used at 2 mM. Note that the ubiquitinylated HIF-1a produced via Lbl treatment does not translocate to the nucleus. Also note the ineffectiveness of succinate.

Figure 10: Immunohistochemical demonstration of HIF-1a activation by branched chain 2-oxoacids

U251 cells were treated with 2 mM doses of the indicated 2-oxoacids for four hours in glucose-free Krebs buffer. Cells were then washed, fixed and stained for HIF-1a protein.

Figure 11: Pyruvate and Oxaloacetate compete for 2-oxoglutarate binding to HIF Prolyl hydroxylases

(A) Human glioma cells express HPH homologues 1, 2 and 3. RT-PCR was performed using specific primers to demonstrate the presence of HPH homologues in the glioma cell lines used to gather most of our data. The pattern of expression seen is similar to those of normal human tissues. (B) HPH bind to immobilized 2-oxoglutarate. This assay is a measure of step B in figure 2. Epoxy-activated Sepharose beads covalently coupled with 2-oxoglutarate were

incubated with *in vitro* translated ^{35}S-labeled HPH homologues in the presence and absence of 250 mM iron sulfate at room temperature and then pelleted by centrifugation. Following four further washes radiolabel associated with the pellets was measured via scintillation counting. More than 50% of the radiolabel bound was iron dependent. (C) Nearly half of the total binding of HPH to the 2-oxoglutarate column could be displaced by 20 mM 2-oxoglutarate but not by 20 mM succinate. (D) Iron dependent HPH binding to immobilized 2-oxoglutarate is displaced by pyruvate (20 mM) and oxaloacetate (20 mM).

Figure 12: Pyruvate and oxaloacetate do not support hydroxylation of HIF-1a ODD peptide

We examined whether pyruvate or oxaloacetate influenced the prolyl hydroxylation of HIF-1a by monitoring the ability of HPH homologues to confer ^{35}S-pVHL binding activity onto a biotinylated19mer peptide containing the key proline564 residue of the HIF-1a ODD (see figure 1). After incubating the peptide with the HPHs and the indicated reagents, ^{35}S-pVHL was added followed by addition of streptavidin-coated beads to pull down the HIF-1a peptide. The reaction was pelleted, the pellet was washed and then solubilized for SDS-PAGE analysis followed by autoradiography to reveal the captured ^{35}S-pVHL. Using *in vitro* translated HPH homologues we first optimized assay conditions with respect to the required HPH substrates and co-factors. (A) 2-OG was absolutely required for activity as shown by this dose curve. Conditions for other reagents were: ascorbate = 2 mM, iron sulfate = 250 μM, DTT = 1 mM. (B) Iron was also absolutely required up to a maximal of about 100 μM. Conditions: ascorbate = 2mM, 2-OG = 2 mM, DTT = 1 mM. (C) Although some activity was seen in its absence, ascorbate dose-dependently enhanced activity. Conditions: 2-OG = 125 μM, iron sulfate = 250 μM, DTT= 1 mM. (D) Under conditions where all other reagents were kept constant as above, 1 mM amounts of Pyr or OAA could not substitute for 100 μM 2-OG in catalyzing proline hydroxylation of HIF-1a peptide by any of the three HPH homologues. In all assays 5 μl (about 20 ng) of enzyme and 1 μg of peptide was utilized.

Figure 13: *In vitro* effects of 2-OG analogs on recombinant HPH activity

HPH activity was assessed via the ^{35}S-pVHL pulldown assay as in figure 11. Activity of *in vitro* translated HPH homologues was determined in the absence and presence of the 2-OG analogs N-oxalylglycine (NOG), pyruvate or OAA at 1 mM. While inhibition by NOG is clearly evident, the effects of pyruvate and oxaloacetate are less consistent at either 5 mM or 25 mM [2-OG].

Figure 14: Oxaloacetate and pyruvate exhibit ascorbate-reversible inhibition of *in vitro* recombinant HPH activity

The ^{35}S-pVHL capture assay used in figure 11 was employed to determine whether OAA or Pyr could act as inhibitors of HPH activity. Conditions employed were: 2-OG = 100 mM, iron sulfate = 20 mM, DTT = 1 mM. Ascorbate concentrations were varied as indicated and OAA or Pyr were added where indicated at 1 mM. Both OAA and Pyr appeared to inhibit the HPH-1 and HPH-2 activity with their effects being more apparent at lower ascorbate doses. HPH-3 did not appear to be sensitive to OAA or Pyr.

Figure 15: Pyruvate or oxaloacetate-induced HIF-1a accumulation is selectively blocked by ascorbate

U87 and U251 glioma cells were treated for four hours in glucose-free Krebs buffer under the indicated conditions. Pyruvate and OAA were included at 1 mM where indicated. (A) Nuclear accumulation of HIF-1a in U87 cells was assessed in nuclear extracts via western blotting. (B) Nuclear accumulation of HIF-1a in U251 cells was analyzed by immunohistochemistry. Note the inhibition of HIF-1 accumulation by ascorbate (100 μM) under pyruvate and oxaloacetate treatment but not under hypoxia. Also note that 2-oxoglutarate (10 mM) was unable to reverse either inducer. Unstimulated U87 cells are not shown in this figure.

Figure 16: Ascorbate reverses the prolonged HIF-1a accumulation by pyruvate and oxaloacetate

(A) Comparison of HIF-1a decay in U251 following induction with either hypoxia or pyruvate. U251 cells were cultured in glucose-free Krebs under hypoxia or with 1mM pyruvate in normoxia for four hours. Following this cells were switched to glucose free Krebs in normoxia and fixed in formaldehyde at the indicated times. Cells were then stained for HIF-1a immunoreactivity. Note the rapid decay of nuclear HIF-1a staining after having been induced by hypoxia versus pyruvate. (B) U87 cell treated for four hours under hypoxia show prominent HIF-1a induction which is completely degraded by thirty minutes of re-oxygenation. U87 cells were treated in glucose-free Krebs buffer with or without 1 mM pyruvate or oxaloacetate. After four hours cells were washed in glucose-free Krebs for various times with or without 100 μM ascorbate being included in the wash. Note that the pyruvate and oxaloacetate-induced HIF-1a accumulation persists for a long time after the inducing agents have been washed away. Inclusion of ascorbate in the wash enhanced the rate of HIF-1a decay.

Figure 17: Inactivation of cellular HPH activity by pyruvate and oxaloacetate

U251 cells were cultured for four hours in glucose-free Krebs buffer with or without the indicated additions. Whole cell extracts were then prepared and used as a source of HPH enzyme to hydroxylate a biotinylated peptide from the HIF-1a ODD containing proline 564. Proline hydroxylation was measured by the ability of streptavidin coated beads to pulldown the hydroxyproline ^{35}S-pVHL complex as in figures 12-14. (A) When cells were treated with 1 mM pyruvate or oxaloacetate there was a marked reduction in the HPH activity of U251 extracts. Inclusion of 100 μM ascorbate during the cell incubation prevented this loss of activity. (B) Similar experiments with hypoxia or DMOG showed no such loss of HPH activity.

Figure 18: 2-oxoacids activate HIF-mediated gene expression in human cell lines

Effective gene expression by HIF not only involves HIF protein stabilization via inhibition of HPH enzymes but also HIF-1 binding to DNA, inhibition of FIH-1 activity, and gene transcription (see figure 1). (A) Glioma cells used in our studies express FIH-1 as assessed using RT-PCR. (B) nuclear extracts from pyruvate treated U87 cells express binding activity for HRE DNA. (C) U87 cells also upregulate mRNA levels of several gene known to be regulated by HIF, such as vascular endothelial growth factor (VEGF), glucose transporter isoform 3 (Glut-3) and aldolase A (Aldo A). Expression of beta-actin, a housekeeping gene not under HIF regulation is not affected by pyruvate. (D) Human Hep3B hepatoma cells express erythropoietin (epo) mRNA and this expression id dose dependently increased by pyruvate. (E) U373 cell were transfected with a green fluorescent protein (GFP) construct under the control of an HIF regulatory element (HRE) containing promoter and then cultured for eight hours in glucose-free medium under the indicated conditions. GFP (green fluorescence) was expressed when cells were treated with 1% oxygen or DFO. Pyruvate also enhanced GFP expression. (F) HRE regulated luciferase was used to demonstrate activation of HIF regulated genes by 2-oxoacid and their analogs. U251 cells stably transfected with a luciferase construct under the control of an HRE containing promoter were cultured for 6h in glucose free Krebs with the following conditions: 1 = control, 2 = 1% oxygen, 3 = pyruvate (2 mM), 4 = OAA (2 mM), 5 = Ethylpyruvate (2 mM), 6 = Glucose (5.5 mM), 7 = DMOG (0.5 mM), 8 = Lactacystin-beta lactone (20 mM). Note the enhanced HRE-luciferase expression by hypoxia, DFO and 2-oxoacids, but not by lactacystin.

Figure 19: HIF-mediated gene expression by the 2-oxoacids pyruvate and oxaloacetate is selectively reversed by ascorbate

U251 cells stably expressing HRE-luciferase were cultured in glucose-free Krebs buffer with the indicated conditions for eight hours. Pyruvate, oxaloacetate, and DMOG were added at 1 mM each. Activation of HRE-luciferase by pyruvate or oxaloacetate is distinguished from that by hypoxia or DMOG by its selective reversal by ascorbate. No reversal was seen with 10 mM 2-oxoglutarate.

Figure 20: 2-Oxoacids activate HIF in brain cells

(A) Primary cultures of rat cerebral cortical neurons grown in Neurobasal media were treated with either 1% oxygen or 3 mM pyruvate for four hours and then assayed for HIF-1a immunoreactivity. Note the increased nuclear accumulation of HIF-1a by both hypoxia and pyruvate. (B) Similar experiments were carried out with primary cultures of rat astrocytes, except that nuclear extracts were prepared and assayed for HIF-1a by western blotting. (C) Ten day old rats were subjected to hypoxia (8% oxygen) or were injected with 2 g/kg pyruvate i.p. Four hours later, rats were sacrificed, their brains were harvested, and nuclear extracts were prepared. HIF-1a levels were determined by western blotting. (D) In a similar experiment ten day old rats were exposed to 0.1% carbon monoxide to produce systemic hypoxia or were injected i.p. with 2 g/kg OAA.

Figure 21: Oxaloacetate preconditioning can protect neurons from oxygen glucose deprivation

OAA preconditioning involved the addition of OAA at different concentrations directly adding it to the medium 48 hours prior to oxygen-glucose deprivation (OGD). Immediately before starting OGD the Neurobasal medium (N/B27) was removed and washed out with phosphate buffered saline. Thereafter, OGD was induced with Krebs buffer without glucose and cells were placed in hypoxia chamber (1% oxygen) for two hours. In control experiments the medium was replaced by regular glucose containing Krebs buffer and the cells were incubated in a normoxic atmosphere of 20 to 21% oxygen. Immediately after OGD, buffers from different treatment groups were removed and replaced with fresh medium, cells were assayed for cell viability 24 hours post insult with MTT reduction. OAA showed protective effects at concentration of 1 mM and this effect was statistically significant (*$p < 0.05$ at 3 mM oxaloacetate treatments).

Detailed Description

General Description

The invention is derived from the discovery that certain endogenous 2-oxoacids are responsible for the regulation of HIF-1 levels under normoxic (20 to 21% oxygen) conditions. Specifically, the endogenous 2-oxoacids pyruvate and oxaloacetate compete for the 2-oxoglutarate binding site in HIF hydroxylating enzymes and then lead to their inactivation. This results in long-lasting HIF-1 accumulation and activation of HIF-1 mediated gene expression, even in the presence of oxygen.

Specific Embodiments

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described.

As used herein, the term "binding" refers to the adherence of molecules to one another, such as, but not limited to, enzymes to substrates, proteins to proteins, transcription factor proteins to DNA, and DNA or RNA strands to their complementary strands. Binding occurs because the shape and chemical nature of parts of the molecule surfaces are complementary. A common metaphor is the "lock-and-key" used to describe how enzymes interact with their substrate.

As used herein, the term "transcription factor" refers to any protein or protein complex that binds to specific regulatory regions of DNA to stimulate gene expression.

As used herein, the term "gene expression" refers to the enhanced production of messenger RNA (mRNA) from DNA, which eventually leads to enhanced protein coded for by the mRNA and to enhanced protein function.

As used herein, the term "hypoxia" refers to oxygen tensions below 5%. Normal air is composed of 20 to 21% oxygen, a condition referred to as "normoxia" in the art.

As used herein, the term "therapeutic agent" refers to any composition, which integrates the core chemical structure of pyruvate and oxaloacetate required for binding to HIF-1a hydroxylating enzymes. Examples include, but are not limited to, methyl-, ethyl-,and

glycerol-esters of pyruvate and oxaloacetate. Other example may include agents that raise pyruvate and oxaloacetate tissue levels by preventing their breakdown.

Methods of Use

Induction of hypoxic adaptation in heart attack or stroke prone or post-heart attack and post-stroke victims. These two conditions are among the leading causes of death and disability in our society. The few medications available for prevention of heart attacks and strokes today include antihypertensive agents, aspirin and other anti-platelet agents and cholesterol lowering drugs. In animal experiments, hypoxic or ischemic preconditioning provides far more prophylactic protection against heart attack and stroke than all of these other approaches. Furthermore, since the pharmacological induction of hypoxia-activated genes represents a novel approach and a distinct mechanism for providing protection against ischemic insults for which there are no competing products. Such an approach would compliment all preexisting approaches. Our approach would also be essential for improving recovery from such insults. The effectiveness of such an approach can easily be determined in pre-clinical cell culture and animal model in which direct comparison is made between the effect of 2-oxoacids and aspirin (or other agents). Stroke or heart attack prone rats could serve as an effective model for this.

Preventive treatment to reduce risk in settings of predictable stroke: cardiac bypass surgery, carotid endarterectomy, deep sea diving. In addition to the prophylactic use of 2-oxoacids and their derivatives in protecting against recurrant strokes or heart attacks, our discoveries could also be utilized for the induction of prophylactic neuroprotection in settings where there is a significant risk of suffering from a stroke. Thus individual who undergo cardiac bypasss surgery or carotid endarterectomy, two of the most common surgical procedures today, suffer a significant incedence of ischemic brain injury. Preloading these patients with 2-oxoacids that induce hypoxia-regulated genes may provide asignificant protection. The effectiveness of such an approach can easily be determined in pre-clinical cell culture and animal model in which direct comparison is made between 2-oxoacid pretreatment and hypoxic preconditioning. Several cell culture and animal models are available for this.

Improvement of glucose metabolism in diabetes. Diabetes also continues to be one of the major medical problems facing our society. In fact, Type 2 diabetes continues to increase in incidence. high blodd glucose is also a risk factor for many other diseases. Nearly half of the 3 dozen or so genes found to be regulated by HIF-1 so far are concerned with enhancing glucose

metabolism. This includes not only the uptake of glucose but also its metabolism via key regulatory enzymes. Currently there is no effective clinical strategy for improving glucose metabolism in diabetic patients and treatment is limited to the use of agents that either enhance insulin secretion or enhance insulin receptor sensitivity. The use of 2-oxoacids and their derivatives to upregulate the expression of glucose transporters and glycolytic enzymes would constitute a novel approach for which there are no competing products. Such an approach would also compliment all preexisting approaches. The effectiveness of such an approach can easily be determined in pre-clinical cell culture and animal model in which direct comparison is made between the effect of 2-oxoacids and other anti-diabetic agents. Mutant mice bred to develop diabetes could serve as an effective model for this. Since oral ingestion of the 2-oxoacids pyruvate or oxaloacetate is harmless to humans, this approach can also be easily tested in clinical trials as an add-on therapy simply by following blood or urine glucose levels.

Neovascularization of ischemic tissue in any form of vascular disease. Recovery from stroke and heart attack may require tissue neovascularization. this may also be the case in many peripheral vascular diseases such as atherosclerosis, vasculitis, phlebitis, or thrombosis. Currently there is no routine approach to parmacologically revascularize issue. Gene therapy approaches that aim to boost tissue levels of vascular endothelial growth factor (VEGF) or fibroblast growth factor 2 (FGF2) are the primary competing technologies, but these ave not yet been effectively realized. The use of 2-oxoacids and their derivatives may represent a novel and effective approach towards this goal. In fact, the ability of enhanced glycolytic metabolism, and of lactate and pyruvate in particular, to induce the elaboration of angiogenic factors and to enhance angiogenesis has been known for over 15 years (18). This powerful effect of 2-oxoacids was recently shown in animal models to produce prominant neovascularization (19). Despite these long-standing observations, our work represents the first mechanistic explanation of this phenomenon since the expression of VEGF as well as the VEGF receptor is regulated by HIF-1 (table 1; figure 18C). In fact our elucidation of this mechanism may lead to the development of far more potent and long acting 2-oxoacid derived drugs that can promote angiogenesis of ischemic tissue. Several animal and cell culture models are now routinely available to test the effectiveness of this approach. These include commercial systems utilizing cultured endothelial cells and animal models to include chick allantoic membrane and mammalian corneal membrane.

Improvement of wound and burn healing. Tissue neovascularization and tissue growth

is crucial for the healing of wounds and burns. By activating HIF-1, the topical application of 2-oxoacids could induce the expression of genes that promote angiogenesis and enhance the growth of connective tissue elements and of epithelial cells. Indeed, hypoxia-regulated gene expression plays a prominant in fetal wound regeneration and adult wound repair (20,21). The activation of HIF-1 represents the key event in turning on these genes. 2-oxoacids such as pyruvate, oxaloacetate, or derivatives there of could easily be prepared into pastes, powders, gels, and lotions or could be incorporated in bandages and applied topically to promote wound and burn healing via HIF-1 activation. Currently there are no competing products that could turn on hypoxia gene expression via topical application. The effectiveness of this application could easily be tested using animal models or clinical trials.

Treatment of anemias. HIF-1 was originally discovered as the transcription factor regulating the expression of the erythropoietin gene. Erythropoietin (EPO) is known to be produced by kidney and liver tissue in response to hypoxia. EPO acts upon the EPO receptor (EPOR) on red blood cell precursors in the bone marrow to bring about a proliferation of red blood cells. EPO is so effective in improving clinical anemias that it is now used routinely in treating a variety of anemias seen in clinical settings. In addition to inducing endogenous EPO production, HIF-1 also induces expression of the transferrin and transferrin receptor genes, which make it possible for red blood cell precursors to turn into mature red blood cells capable of carrying oxygen. Thus an activator of HIF-1 would represent a more effective anti-anemic agent. The 2-oxoacids pyruvate and and oxaloacetate and derivatives thereof could be given orally or parenterally to improve anemia via HIF-1 activation (see figures 18D and 20E). Since oral ingestion of the 2-oxoacids pyruvate or oxaloacetate is harmless to humans, this approach can also be easily tested in clinical trials simply by following blood hematocrit levels.

Acclimation to high altitudes. High altitudes atmospheres have the same % composition of oxygen as low altitudes. However, due to the lower pressure, high altitude air has fewer gas molecules overall and thus lower oxygen levels. Symptoms of high altitude sickness such as headaches, hyperventilation, fatigue and death are due to insufficient oxygen delivery to tissues. Thus insufficient oxygen at high altitudes requires that mammals adapt their physiology in order to survive. The acclimation of mammals to high altitudes is primarily governed by an acute increase in ventilation as well as a sustained increase in HIF-1 mediated gene expression (2). Such genes facilitate mammalian physiology at high altitudes by improving blood oxygen carrying capacity and tissue oxygen delivery while simultaneously

improving the oxygen-independent glucose metabolism of the body's cells. The major approach currently available for effectively enhancing adaptation of humans to low oxygen is to ascend slowly thus allowing HIF-1 mediated gene expression to ensue. The prophylactic use of pyruvate or oxaloacetate and their derivatives may markedly improve and facilitate high altitude acclimation. Although this application has a limited commercialization potential, it may find significant utility amongst travelers that visit high altitudes or military or other personnel that may need to rapidly ascent into areas of low oxygen levels.

Smoke inhalation prophylaxis. Although firefighters do not encounter high altitudes routinely, they are at risk for acute bouts of unexpected hypoxia due to smoke inhalation and carbon monoxide toxicity. Prophylaxis with pyruvate or oxaloacetate or derivatives thereof may markedly reduce the chances of such individuals suffering hypoxic injury. Since oral ingestion of the 2-oxoacids pyruvate or oxaloacetate is harmless to humans, this approach can also be easily tested in clinical trials and may improve the health of cigarette smokers.

Asthma attack, seizure, and cardiac arrythmia prophylaxis. As in induction of hypoxic adaptation in heart attack or stroke, patients with asthma, epilepsy, or cardiac arrythmias are at risk for acute bouts of tissue hypoxia. These conditions can potentially lead to significant hypoxic or anoxic injury. Prophylaxis with pyruvate or oxaloacetate or derivatives thereof may markedly reduce the chances of such individuals suffering hypoxic injury. Since oral ingestion of the 2-oxoacids pyruvate or oxaloacetate is harmless to humans, this approach can also be easily tested in clinical trials.

Athletic performance improvement. No population has capitalized upon HIF-1 mediated gene expression more that athletes who compete in highly demanding aerobic sports. In fact, every country's Olympic training centers are located at high altitudes to take advantage of the improvements in physiology that are induced by hypoxia. The hypoxia induced physiological changes described above allow more efficient use of oxygen and also allow the exercising body to utilize anaerobic fuels more efficiently. The result is greater endurance during demanding aerobic exercise or competition. Pyruvate has been used by athletes for a long time during aerobic exercise with the belief that this may provide more metabolic fuel to enhance performance. However, the previously unknown insights offered by our findings (see figure 8) suggest that this strategy is flawed. Highly aerobic exercise results in acute tissue hypoxia due to an enhanced demand for oxygen in the face of unchanging supply. Indeed, pyruvate and lactate accumulate significantly during exercise due to their inadequate

metabolism by oxygen-requiring reactions. Thus there is plenty of pyruvate accumulated during an acute bout of highly demanding exercise. What requires replenishing is oxygen and not pyruvate. Alternatively the changes in cell metabolism and improvements in tissue function induced by chronic hypoxia actually lower the tissue demand for oxygen. In addition, the improved oxygen carrying capacity of the blood and improved tissue blood capillary density provoked by hypoxia may be significant factors in improving athletic performance. These physiological changes take days or weeks to express themselves and are initiated via HIF-1 regulated gene expression (2). Thus, effective use of pyruvate and or oxaloacetate for improving athletic performance should focus not on their obvious role as fuel sources but rather on our discovery of their non-obvious role as HIF-1 activators. Thus chronic ingestion of pyruvate, oxaloacetate, or derivatives thereof by athletes during training and prior to competition could be adjusted to maximize long term changes in HIF-1 mediated gene expression. The increasing clandestine use of EPO by athletes who wish to improve their athletic performance suggests that there is a potentially large market for use of the safer 2-oxoacid derivatives for improving athletic performance. Currently, there is no other legal alternate strategy to achieve this other than high altitude training.

Improving survival of prematurely born infants. Premature birth has a high degree of association with many diseases in subsequent adult life. The development of proper oxygen homeostasis is crucial for life and activation of HIF-1 is crucial for this to happen (1). Indeed, HIF-1a knock out mice die *in utero*. Administartion of pyruvate, oxaloacetate or derivatives thereof to expectant mother at high risk for premature delivery may induce HIF-1 in fetal tissues and accelerate the development of proper oxygen homeostasis. This approach may also be beneficial in preventing stroke-like episodes from pregnancy-associated eclampsia.

Preservation of donor organs prior to transplant. The use of pyruvate, oxaloacetate or derivatives thereof to induce HIF-1 in the organs of tissue donors prior to harvesting and also the addition of these agents to donated organ storage solution may improve the hypoxic survival of organs during the time that they are not adequately perfused.

Improvement of immune function. Immune activity has recently been shown to be dramatically reduced upon knockout of the HIF-1a gene (22). Administration of pyruvate and oxaloacetate to immunodeficient individuals could improve outcome form a variety of immunodeficient diseases such as AIDS.

Examples

Recently, it was determined that human cells lines to display basally elevated levels of HIF-1a even under normoxic conditions (20% oxygen) (10). The level of this basal HIF-1a expression varied with the specific cell line studied. Further exploration revealed that the differential basal expression of HIF-1 was a function of the different culture media that were being used to propagate the specific cell lines. Cells grown in media containing high glucose or added pyruvate appeared have detectable levels of HIF-1a under normoxia. In order to resolve the biochemical mechanisms underlying this phenomenon, we studied the human glioma cell line U-87 under conditions of carefully defined culture media. Thus we studied these cells while culturing them in freshly prepared Krebs buffer, all components of which were known to us. We found a time-dependent elevation of HIF-1a levels in these cells upon changing their media with fresh Krebs buffer (figure 4). Systematic removal of each component of the Krebs buffer revealed that the key component that led to accumulation of the HIF-1a protein was glucose. Thus, no increase in HIF-1 levels was seen in glucose-free Krebs, while glucose dose-dependently increased HIF-1a levels. Furthermore, the ability of glucose to stimulate HIF-1a could not be mimicked by the non-metabolizable glucose analog 2-deoxyglucose. Thus a metabolite of glucose was responsible for the accumulation of HIF-1a. Hypoxia and DFO, two known activators of HIF-1, could however still upregulate HIF-1a protein in the absence of glucose. these results demonstrate that the glucose-mediated effects represented a novel mechanism distinct from those previously recognized.

To precisely define the glucose metabolite mediating HIF-1a accumulation, we utilized pharmacological inhibitors of glycolysis as well as the direct addition of different glucose metabolites to cells (figure 4). Iodoacetamide, an inhibitor of glyceraldehyde phosphate dehydrogenase (GAPDH) completely blocked the ability of Krebs buffer to stimulate HIF-1a accumulation. Cinnamate, an inhibitor of pyruvate and lactate transport across mitochondrial and plasma membranes, did not prevent the effect of Krebs buffer on HIF-1a accumulation. These results narrowed down the responsible glucose metabolite to the steps after GAPDH. Addition of pyruvate and lactate in glucose-free Krebs was then found to activate HIF-1a directly while several pyruvate metabolites such as citrate, 2-oxoglutarate, succinate, and alanine were without effect. Lactate and pyruvate are highly produced by human cell line such as the U87 glioma cells that we primarily studied (figure 5). Lactate and pyruvate

are also intercovertible via the enzyme lactate dehydrogenase (LDH). Glucose metabolism to pyruvate raises the cellular NADH/NAD ratio while pyruvate conversion to lactate lowers this ratio. To rule out the possibility that a change in NADH or NAD levels was responsible for elevation of the HIF-1a protein we utilized cells permeabilized with mild detergent (digitonin) treatment. these preparations were able to induce HIF-1a by all known mediators. Direct addition of 3 mM NAD or NADH had no effect on HIF-1a levels, demonstrating that NADH/NAD ratios were not responsible for the HIF-1a activation by glucose metabolism. We utilized the LDH inhibitor oxamate to more specifically implicate pyruvate in HIF-1a activation. Oxamate blocked the ability of lactate to stimulate HIF-1a accumulation while potentiating the effect of pyruvate. These results demonstrated thet although lactate can stimulate HIF-1a accumulation, it must first be converted into pyruvate. Thus pyruvate was the major glucose metabolite responsible for stimulating HIF-1a accumulation. Pyruvate appears to enhance the accumulation of HIF-1a by inhibting its degradation in a manner resembling the inactivation of the HPH enzymes. This was demonstrated in fiigure 5 by the observation that pyruvate maintained elevated HIF-1a levels in the absence of prtein synthesis. Normally, HIF-1a has a very short half life and is degraded within minutes of being synthesized in the presence of oxygen and iron.

In evaluating other cellular metabolites we found that the structural requirements for HIF-1 activitating metabolites were quite specific. We found that oxaloacetate, a major metabolite of pyruvate and a Krebs cycle intermediate, is also a potent and effective inducer of HIF-1a protein (figure 6). Oxaloacetate and pyruvate can be interconverted via several metabolic routes. Despite this, both of these glucose metabolites appear sufficient to potently induce HIF-1a levels in many cell types.

The structural diagrams in figure 7 are also revealing from the standpoint of future drug development based upon 2-oxoacids. We have shown that while oxaloacetate can activate HIF-1, succinate and 2-oxoglutarate cannot. This points out the importance of the 2-oxo group in mediating HIF-1 activation, yet also shows the importance of appropriately positioned groups at the 4 and 5 positions. That pyruvate can activate HIF-1 shows that the minimal features we have detemined to be required so far in activating HIF-1 are the 2-oxo group and a methyl group at the 3 position. Citrate, which has a carboxl group at position 3 is ineffective as is malate. Simple biochemical derivative of pyruvate and acetate can be screened for the activation of HIF-1 in glucose-free media exactly as we have demonstrated above. This may

allow for the development of simple drugs far more potent and stable than pyruvate for regulating hypoxic gene expression. Thus, our elucidation of the regulation of HIF-1 by small 2-oxoacids can be utilized to develop drugs that induce physiological responses, which improve survival under hypoxia. Note that the ethyl- and methylpyruvate derivatives that we have identified as HIF-1 activators are already being proposed for use in other clinical applications (11,12)

In order to investigate the mechanism of action of the 2-oxoacid HIF inducers that we identified we first determined their effects upon cellular ATP levels. As shown in figure 8A, during the four to eight hour culture periods used in our studies, addition of glucose or glucose metabolites to our glucose-free buffer does not have significant effects upon cellular ATP levels. Note that the effects of glucose, pyruvate, and oxaloacetate on ATP levels are not significantly different from those of 2-oxoglutarate and succinate dspite their dramatically different effects on HIF-1a induction. Moreover, direct addition of ATP to permeabilized cels did not raise basal HIF-1a levels (Figure 8B). These data point to a mechanism of HIF induction distinct from a change in cellular phosphorlylation potential. As shown in the scheme in figure 1, cellular levels of HIF-1a can be elevated by blockade of proteasomal activity, ubiquitinylation, or prolyl hydroxylation. Inhibition of the proteasome by lactacystin beta-lactone (Lbl) leads to accumulation of HIF-1 a in its polyubiquitinylated form which generally appears as a smear of higher molecular weight species on western blots. This polyubiquitinylated form does not translocate to the nucleus and does not activate gene transcription. Figure 9A shows that in whole cell extracts of U87 cells treated in Krebs buffer, HIF-1a induced by glucose or pyruvate has a molecular weight similar to that seen with induction by the HPH inhibitors hypoxia and desferrioxamine (DFO). The characteristic high molecular weight smear of poly-ubiquitinylated HIF-1a is only seen with lactacystin treatment. Cellular accumulation and nuclear translocation of HIF-1a can also be studied via immunohistochemistry. For this purpose we utilized U251 cells which are more adherent to cell culture dishes that the U87 cells. As shown in figure 9B, glucose, pyruvate, and oxaloacetate promote nuclear HIF-1a accumulation similar to hypoxia and DFO but are distinguished from lactacystin which only promotes cytosolic HIF-1a buildup. Succinate does not affect HIF-1a accumulation. Using this assay in U251 cells we also determined that endogenous branched chain 2-oxoacids can promote HIF-1a accumulation (figure 10).

These data, together with the structural profile presented in figure 7 strongly

suggested that the endogenous 2-oxoacid HIF-1a inducer may work by competing for the 2-OG binding site in HPHs in a manner similar to NOG or DMOG (see figure 2). We determined that all three known human HPH homologues were expressed wihin the cells we were studying (figure 11A). In order to investigate whether pyruvate or oxaloacetate could compete with the 2-OG binding site on HPH, we prepared an affinity column with 2-OG immobilized onto sepharose beads. We also prepared ^{35}S-labeled HPH homologues from expression plasmids using the rabbit reticulocyte culture system (13). The 2-OG column allowed us to investigate the binding of ^{35}S-HPH as well as potential competitors of binding. This approach, which monitors step B in figure 2 has been used previously (14). As shown in figure 11B all three ^{35}S-labeled HPH homologues bound to the immobilized 2-OG with more than half the binding showing a requirement for iron. Using ^{35}S-HPH-1 we also showed that its substrate 2-OG could displace this binding while the endproduct succinate could not (figure 11C). Using the iron dependent binding of ^{35}S-HPH we showed that both pyruvate and oxaloacetate could indeed compete for the 2-OG binding site (figure 11D).

To directly evaluate the action of pyruvate and oxaloacetate on HPH activity (step D in figure 2) we utilized the commonly used ^{35}S-pVHL pulldown assay. This assay monitors the ability of HPH homologues to confer ^{35}S-pVHL binding activity onto a biotinylated 19mer peptide containing the key proline564 residue of the HIF-1a ODD (see figure 1). After incubating 1 μg amount of the peptide with *in vitro* translated HPH and the indicated reagents, ^{35}S-pVHL was added followed by the addition of Streptavidin-coated beads to pull down the HIF-1a peptide. The reaction was pelleted, and the pellet was washed and solubilized for SDS-PAGE analysis followed by autoradiography to reveal the captured ^{35}S-pVHL. Using each of the *in vitro* translated HPH homologues we first optimized assay conditions with respect to the required HPH substrates and co-factors. This optimization is shown for HPH-1 in figure 12A to C. Both 2-OG and iron were absolutely required for activity and although some activity was seen in its absence, ascorbate was also found to dose-dependently enhance activity. (D) Under conditions where all other reagents were kept constant (ascorbate = 200 μM, ferrous sulfate = 100 μM), 1 mM amounts of Pyr or OAA could not substitute for 100 μM 2-OG in catalyzing proline hydroxylation of HIF-1a peptide by any of the three HPH homologues.

The ability of pyruvate and oxaloacetate to compete for the 2-OG binding site along with their inability to catalyze HIF-1a prolyl hydroxylation supported their potential role as 2-OG antagonists. Therefore, we examined their ability to inhibit 2-OG catalyzed HIF-1a

hydroxylation using the ^{35}S-pVHL pull down assay. However, compared to the well-known HPH inhibitor N-oxalylglycine (NOG) we saw no inhibition by pyruvate little inhibition by oxaloacetate (figure 13). The assay conditions we utilized for this assay as per the literature are optimized to give ideal HPH activity. This includes the use of ascorbate at 2 mM levels so as to avoid the syn-catalytic inactivation described above. Cultured cells, on the other hand, do not routinely have extra ascorbate added to their media. We suspected that the discrepancy that we observed between the robust ability of pyruvate and oxaloacetate to induce HIF-1a accumulation with their poor ability to inhibit *in vitro* HPH activity resulted from inclusion of ascorbate in the *in vitro* assay. By varying the amount of ascorbate in the *in vitro* assay we indeed revealed an inhibitory effect of pyruvate on HPH-1 and HPH-2 activity that appeared to be ascorbate sensitive (figure 14).

Interestingly, HPH-3 activity appeared to be insensitive to pyruvate or oxaloacetate. HPH-2 has been reported to constitute the major HPH activity of most cells. We therefore evaluated the ability of ascorbate to prevent HIF-1a accumulation by pyruvate and oxaloacetate in living cells. As shown in figure 15, inclusion of 100 μM ascorbate in the cultured cell experiments lead to complete inhibition of HIF-1a accumulation by pyruvate and ascorbate but not by hypoxia. Since ascorbate is known to reactivate the 2-oxoglutarate dependent dioxygenases following their syn-catalytic inactivation (figure 2E and F), our results suggested that pyruvate and oxaloacetate bind to HPHs and then inactivate them in an ascorbate reversible manner. The ascorbate sensitive inactivation is highly variable amongst family members of the 2-oxoglutarate dioxygenases and cannot be predicted without empirical data. To test this possibility we compared the reversibility of HIF-1a accumulation following induction with either hypoxia, pyruvate, or oxaloacetate. The rationale for these experiments stems from the well-known reversible inhibition of HPH activity by hypoxia (see figure 1). Thus HIF-1a accumulation and HIF activation during hypoxia is rapidly reversed upon re-oxygenation. Figure 16A shows that the HIF-1a accumulation induced by hypoxia in U251 cells does indeed undergo a rapid decay upon re-oxygenation with no nuclear protein being detectable after 30 minutes of re-introducing oxygen. On the other hand, pyruvate induced HIF-1a accumulation persists well past 40 minutes after washing out the pyruvate. Similar results were seen in U87 cells via western blot analysis of nuclear extracts (figure 16B) with oxaloacetate. To investigate whether the 2-oxoacid induced persistence in HIF-1a accumulation was due to HPH inactivation we repeated this experiment with 100 μM

ascorbate added to the wash buffer. As shown in figure 16C, ascorbate markedly enhanced the decay rate of HIF-1a.

To directly determine whether HPH activity had been inactivated by pyruvate or oxaloacetate treatment, we evaluated the ability of U251 cell extracts from pyruvate or oxaloacetate treated cells to hydroxylate HIF-1a peptide using the ^{35}S-pVHL pulldown assay (15). In these experiments, ascorbate was omitted from the *in vitro* portion of the assay. As shown in figure17A, pyruvate and oxaloacetate pretreatment of cells clearly reduced HPH activity of cell extracts while the presence of ascorbate during the cell incubation period prevented this inhibition. No such pretreatment-induced inhibition was seen with hypoxia or DMOG (figure 17B).

Effective gene expression by HIF not only involves HIF protein stabilization via inhibition of HPH enzymes but also HIF-1 binding to DNA, inhibition of FIH-1 activity, and gene transcription. The human gliomas that we utilized for most of our studies all express mRNA for FIH-1 (figure 18A). Furthermore, incubation of U87 cells under normoxia with pyruvate for four to six hour results in accumulation of HIF regulatory element DNA binding activity (figure 18B), and enhanced expression of several HIF regulated mRNA (figure 18C). In addition, pyruvate treatment of human Hep3B cells, which produce the well-known HIF regulated gene erythropoietin (Epo), resulted in a dose dependent increase in Epo levels (figure 18D). These data imply that pyruvate and perhaps other 2-oxoacids can also inhibit FIH-1 and fully activate the HIF signaling pathway shown in figure 1. To directly assess activation of gene containing an HRE regulated promoter by the glucose metabolites pyruvate and oxaloacetate, we used U373 glioma cells transfected with an HRE-green fluorescent (HRE-GFP) construct that responds to HIF activation. Hypoxia, DFO, and pyruvate all activated HRE-GFP expression as shown by the buildup of cellular fluorescence (figure 18E). In addition, U251 cells that were stably transfected with an HRE-luciferase construct also showed prominent activation of luciferase gene expression by hypoxia, pyruvate oxaloacetate and the ethyl- and methyl-pyruvate derivatives (figure 18F). Using the same HRE-luciferase expressing U251 cells we also were able to show that activation of HIF-dependent gene expression by pyruvate and oxaloacetate is distinguished from that by hypoxia or DMOG by its reversibility with ascorbate (figure19).

Since most of the data presented here were obtained from experiments conducted with human cancer cell lines, we sought to determine whether pyruvate or oxaloacetate could

activate the HIF pathway in normal cells and tissues. We therefore prepared primary cultures of rat cerebral cortical neurons and astrocytes and subjected these cells to a similar analysis as that for the cell lines described above. As shown in figure 20A rat neurons accumulate HIF-1a nuclear immunoreactivity upon four hours exposure to either 1% oxygen or to 3 mM pyruvate. Similarly, primary cultures of rat cerebrocortical astrocytes were also shown to induce HIF-1a upon treatment with hypoxia or with pyruvate (figure 20B). In order to determine the ability of pyruvate and oxaloacetate to activate HIF-1a *in vivo* we injected ten day old rats with intraperitoneal 500 mg/kg-doses of either pyruvate or oxaloacetate. We also subjected littermates to whole body hypoxia with either 8% oxygen or 0.1% carbon monoxide in air. Both of these paradigms have been shown to produce significant hypoxia and HIF-1 activation. Following four hours of each respective treatment, we harvested the animals' brains and prepared nuclear extracts for HIF-1 western blot analysis.

We also harvested kidneys for analysis of erythropoietin mRNA expression. As shown in figure 20C and D, rat brain displayed an increase in HIF-1a immunoreactivity following either hypoxia, pyruvate injection or oxaloacetate injection. Figure 20E shows that renal erythropoietin gene expression was also stimulated by either hypoxia or oxaloacetate treatment. These results demonstrate the utility of using HPH (and presumably FIH-1) inactivating 2-oxoacids to regulate HIF-mediated gene expression.

We have also completed work aimed at demonstrating beneficial physiological outcome from 2-oxoacid induced gene expression. One of these efforts involves the use of 2-oxoacids for hypoxic preconditioning. By inducing cytoprotective HIF-activated genes, 2-oxoacids such as pyruvate and oxaloacetate may be able to reduce the risk of stroke in elective cardiac or carotid surgery, lower ischemic bowel injury following gastrointestinal surgery, and also enhance the grafting efficiency of transplanted organs. To demonstrate the feasibility of such an approach, we have utilized a neuronal cell culture model of ischemic preconditioning. In this model, primary rat neuronal cultures are exposed to sublethal periods of oxygen and glucose deprivation (OGD), thereby mimicking ischemia. This brief OGD period is followed by return of cells to their regular culture conditions. A subsequent lethal period of OGD is then applied and the survival of OGD-preconditioned versus naïve cells is assessed via various cell survival assay to include the routinely used MTT reduction assay (16). Recently, pharmaceutical efforts to induce ischemic or hypoxic preconditioning have been pursued in order to avoid the risk of exposing individuals to sublethal ischemia or hypoxia. One such

recent effort has utilized the HIF-induced gene product erythropoietin, pretreatment with which shows remarkable OGD neuroprotection (17). We utilized the pretreatment paradigm used in this erythropoietin study to test whether oxaloacetate pretreatment could improve neuronal survival during OGD. The basic papradigm is shown in figure 21A. Rat cerebral cortex neurons are cultured for eight days in Neurobasal medium (N/B27). At that point oxaloacetate (OAA) or vehicle is added to the culture medium and the cells cultured for an additional 2 days. Following this period of treatment, neurons were made to undergo a two hour period of oxygen-glucose deprivation in which their media was replaced with a an isotonic salt solution lacking glucose. The neurons were also placed in an environment of 1% oxygen. This OGD paradigm results in significant delayed death of neurons one day later. As shown in figure 21B, pretreatment of neurons with 3 mM OAA improves survival in this paradigm. These results suggest that 2-oxoacids such as pyruvate and OAA that are capable of inducing HIF by inactivating HPHs can be used to induce hypoxic gene expression for therapeutic purposes.

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Table 1
Target genes known to be upregulated by hypoxia via the transcription fact r HIF-1

Adenylate kinase 3
α_{1B}-adrenergic receptor
Adrenomedullin
Aldolase A
Aldolase C
Carbonic anhydrase 9 (CA9)
Caeruloplasmin
Endothelin-1
Enolase 1
Erythropoietin
Glucose transporter 1 (GLUT1)
Glucose transporter 3 (GLUT3)
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
Haeme oxygenase 1
Hexokinase 1
Hexokinase 2
Insulin-like growth factor 2 (IGF2)
IGF binding protein 1
IGF binding protein 2
IGF binding protein 3
Lactate dehydrogenase A (LDH-A)
Nitric oxide synthase 2
NIP3
p21
$p35^{srj}$
Phosphofructokinase L (PFK-L)
Phosphoglycerate kinase 1 (PGK-1)
Plasminogen activator inhibitor 1
Prolyl-4-hydroxylase α(I)
Pyruvate kinase M (PK-M)
Transferrin
Transferrin receptor 1
Transforming growth factor β3
Triosephosphate isomerase (TPI)
Vascular endothelial growth factor (VEGF)
VEGF receptor FLT-1

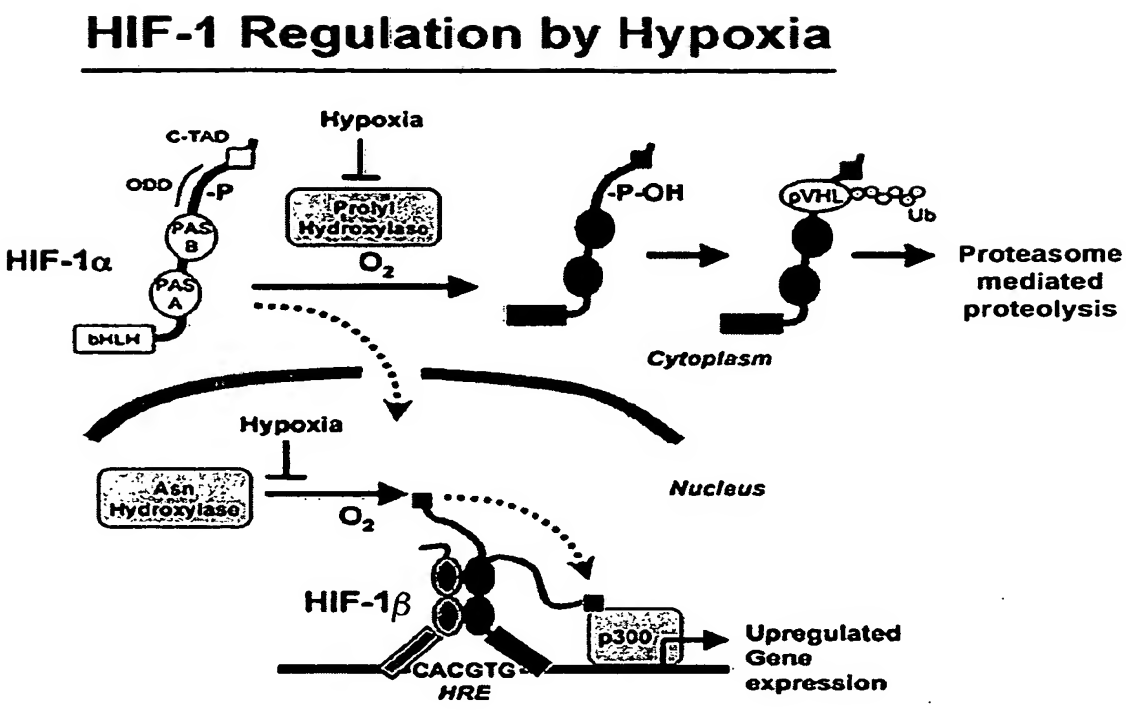

FIG. 1
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Activation of Hypoxia-Inducible Gene Expression
Attorney Docket 044508-5008

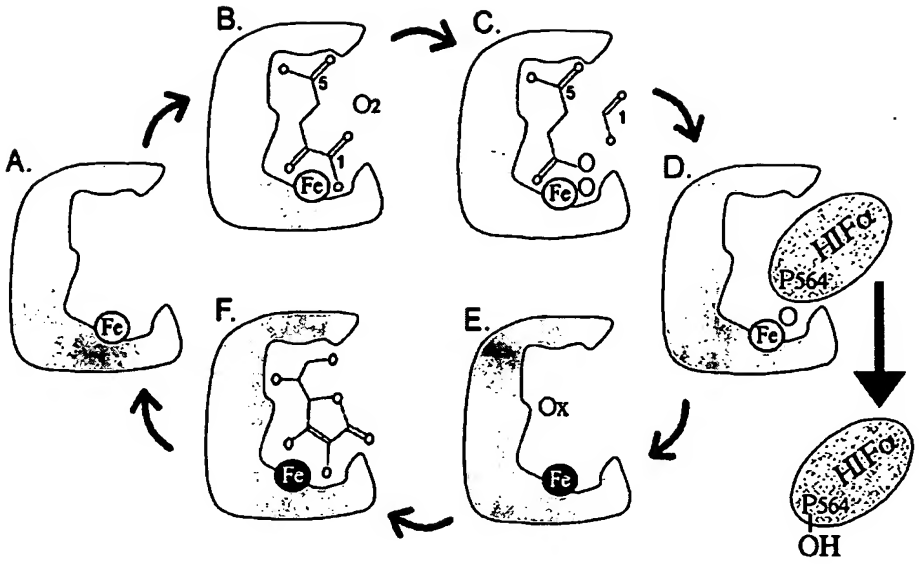

FIG. 2
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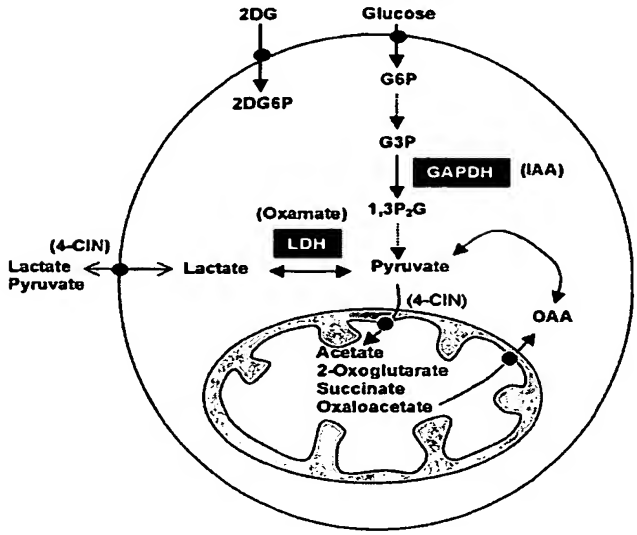
A. Abbreviated diagram of oxygen-independent glucose metabolism and relevant inhibitors.



B. Structural comparisons of key glucose metabolites.

	2-OG	Succ	OAA	Pyr
1	COOH	COOH	COOH	COOH
2	C=O	CH_2	C=O	C=O
3	CH_2	CH_2	CH_2	CH_3
4	CH_2	COOH	COOH	
5	COOH			

FIG. 3
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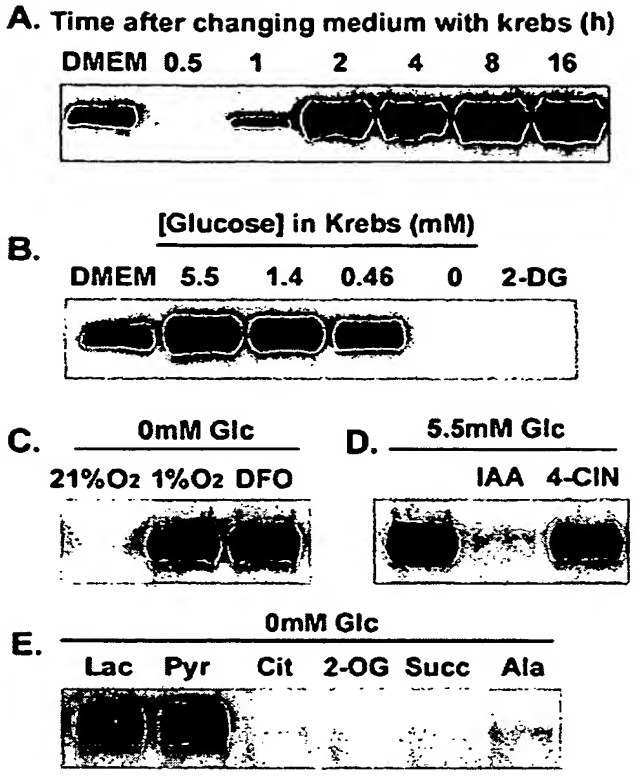

FIG. 4
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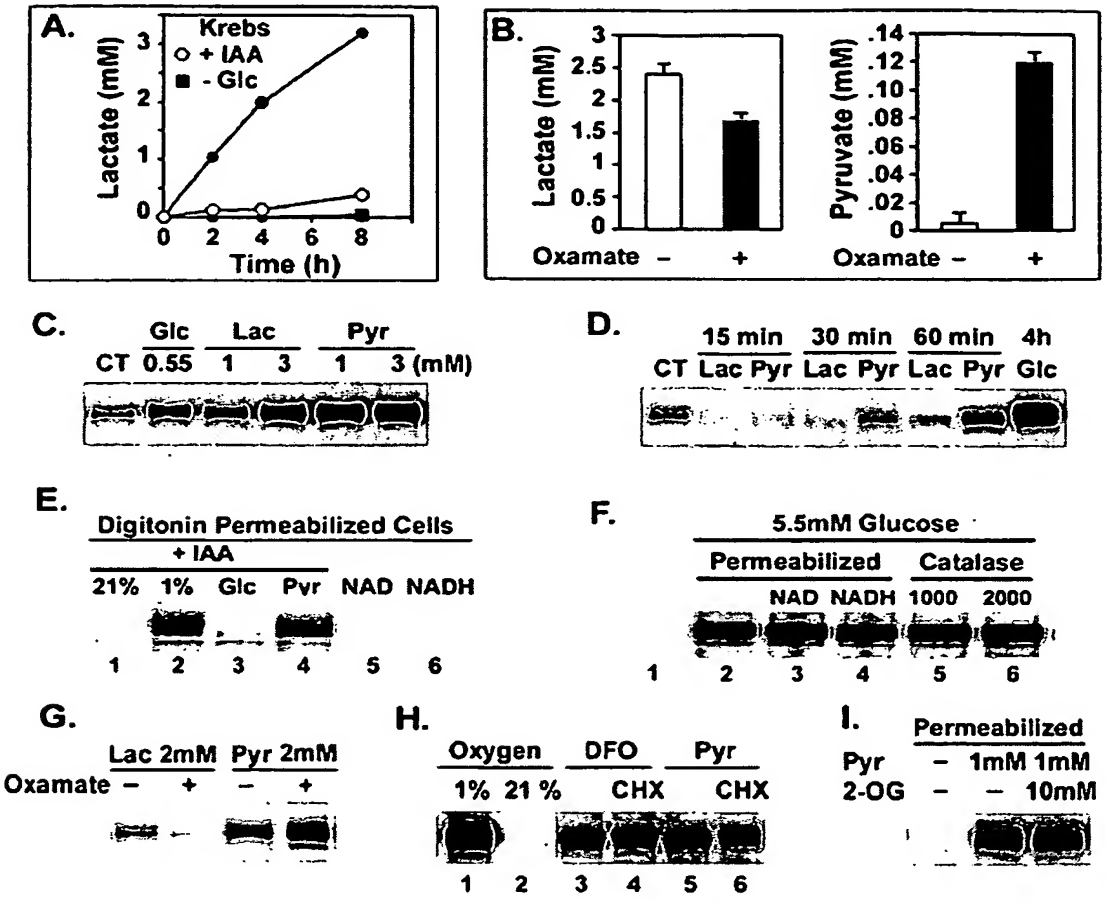

FIG. 5
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Attorney Docket 044508-5008

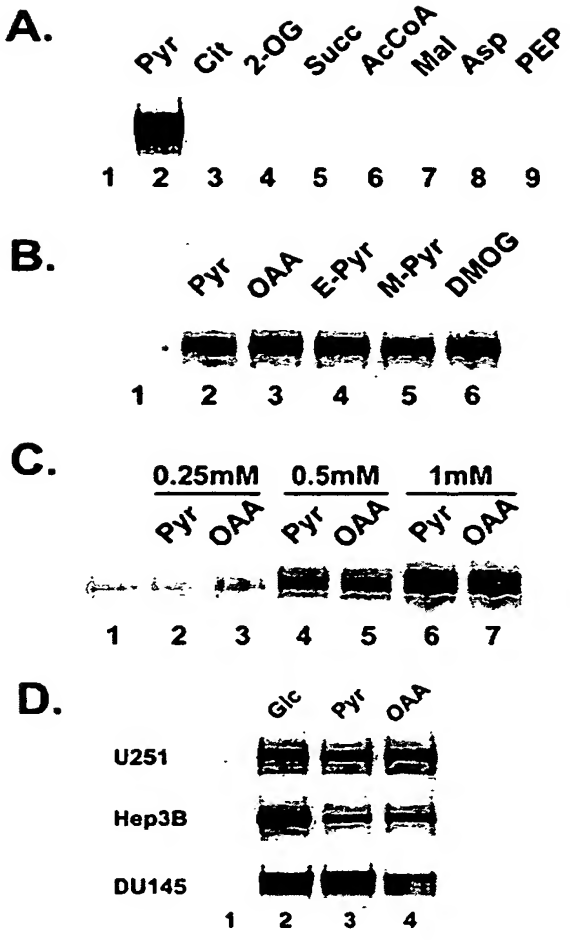

FIG. 6
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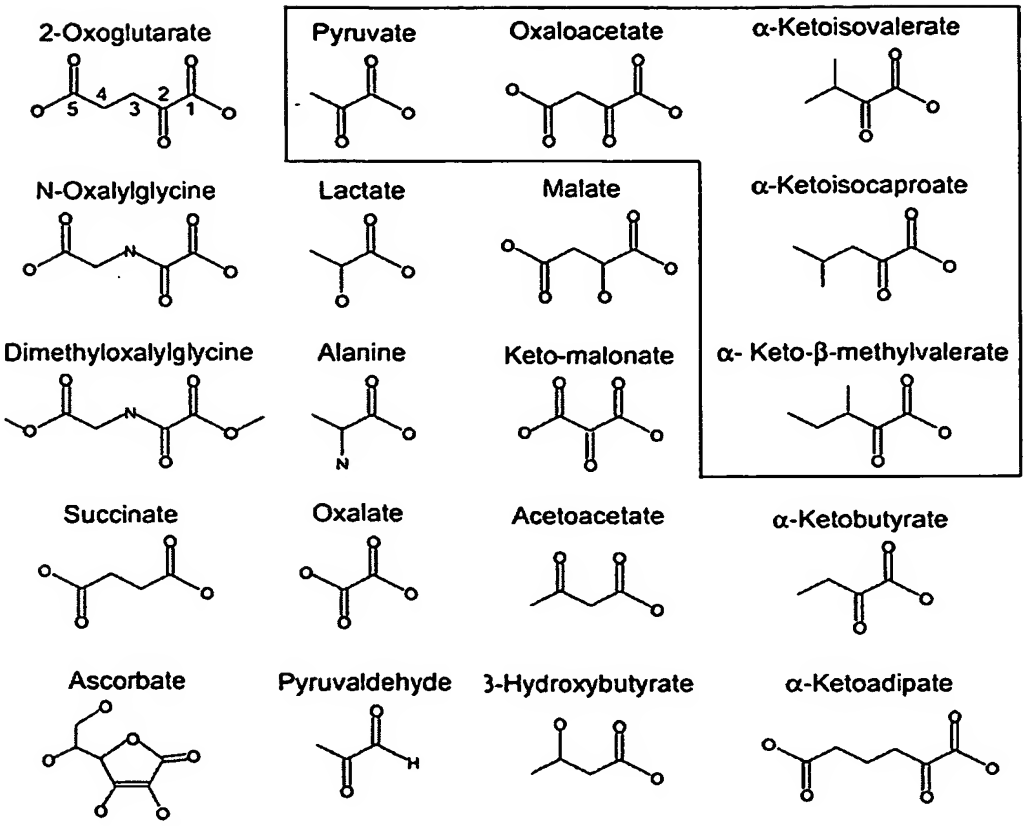

FIG. 7
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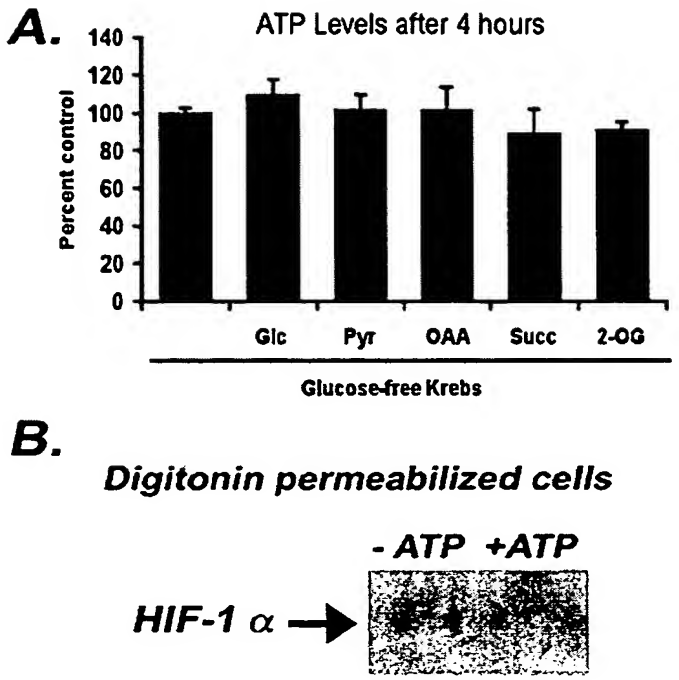

FIG. 8
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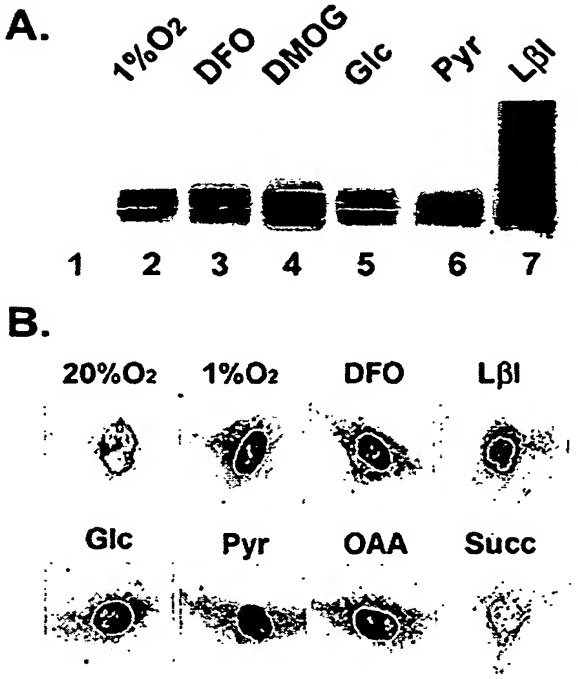

FIG. 9
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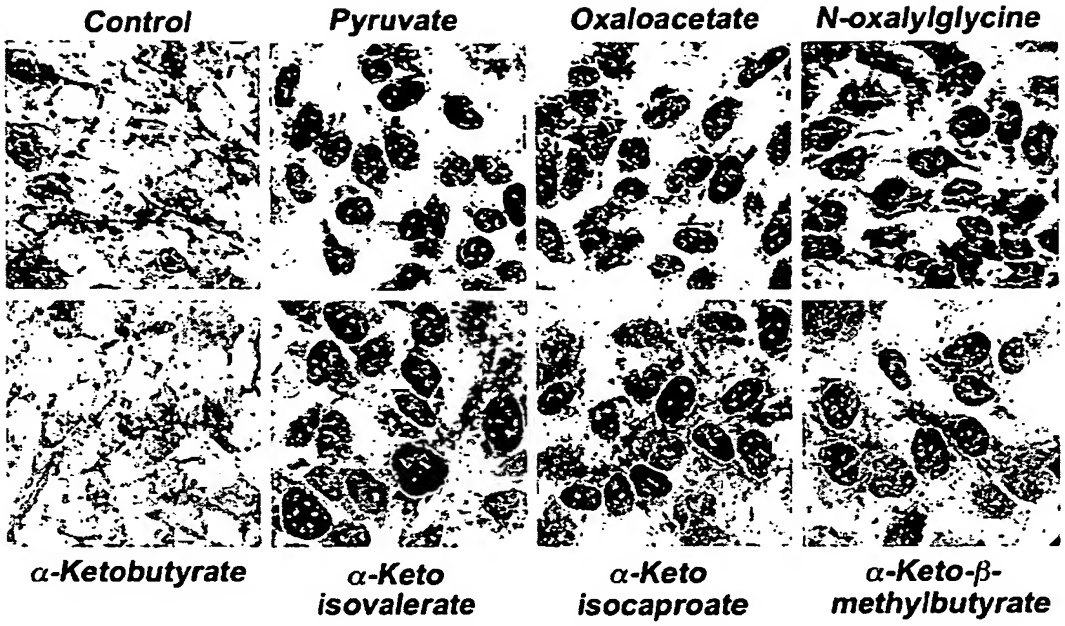

FIG. 10
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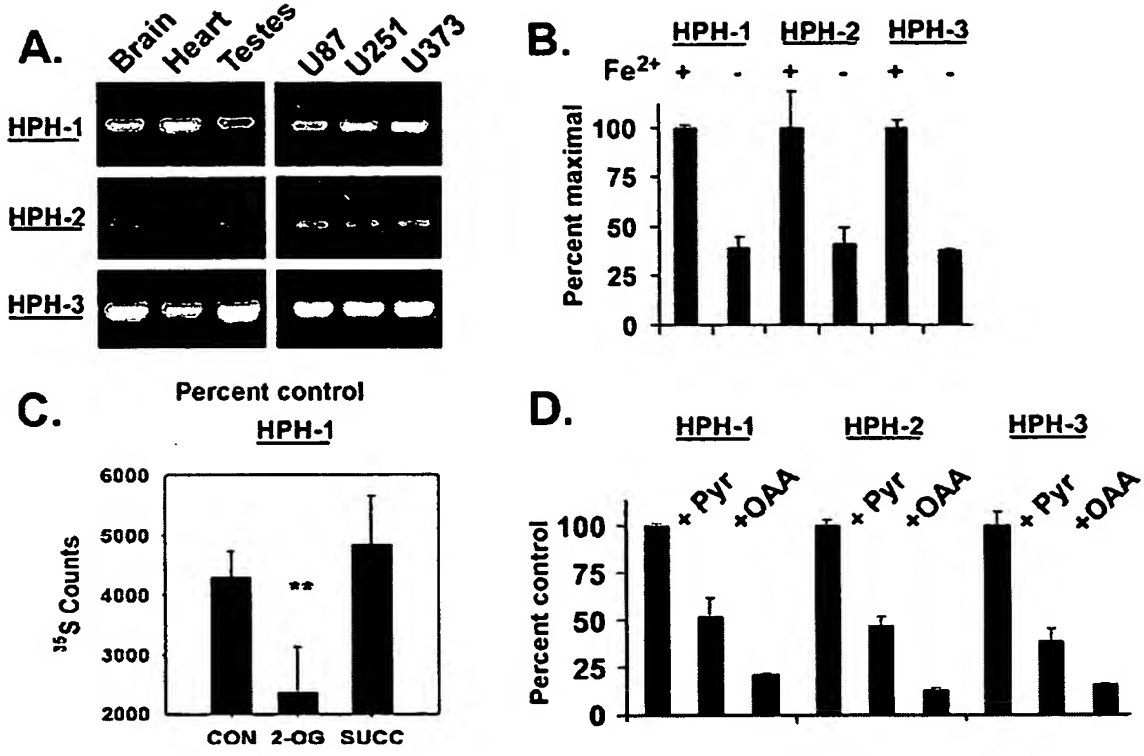

FIG. 11
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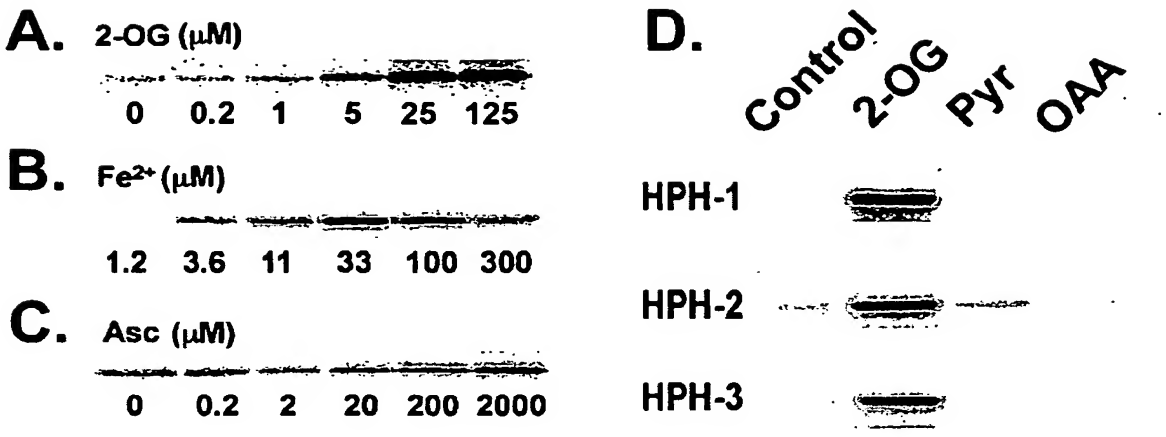

FIG. 12
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Activation of Hypoxia-Inducible Gene Expression
Attorney Docket 044508-5008

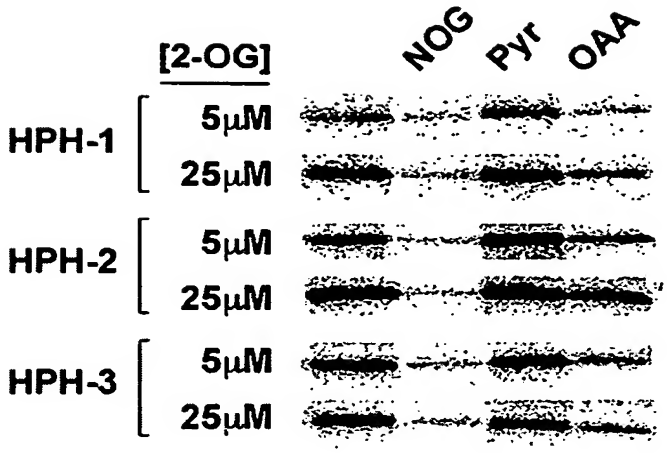

FIG. 13
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Activation of Hypoxia-Inducible Gene Expression
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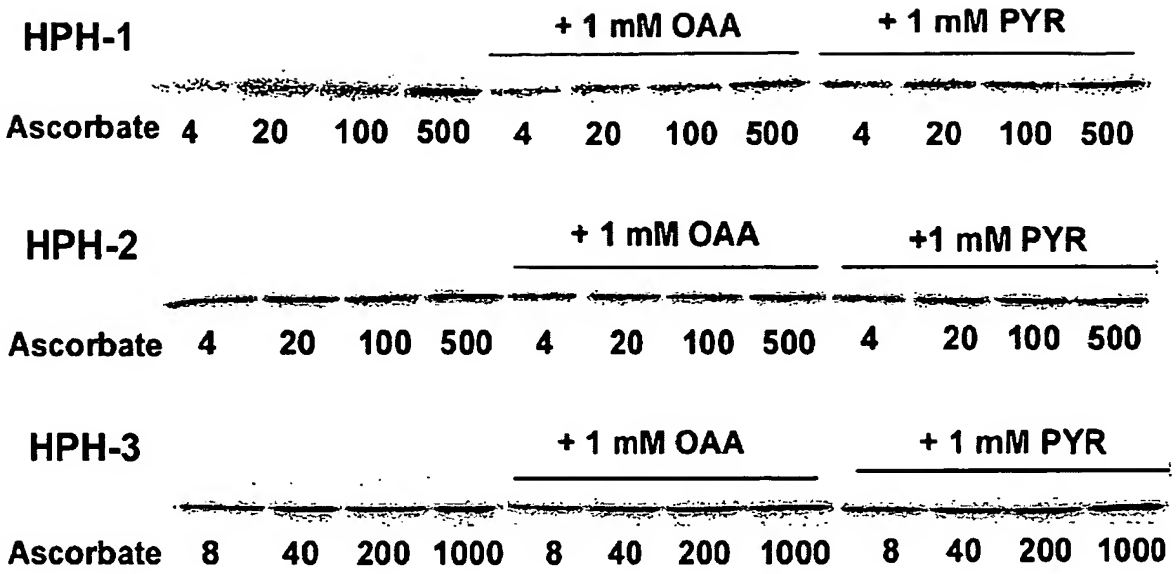

FIG. 14
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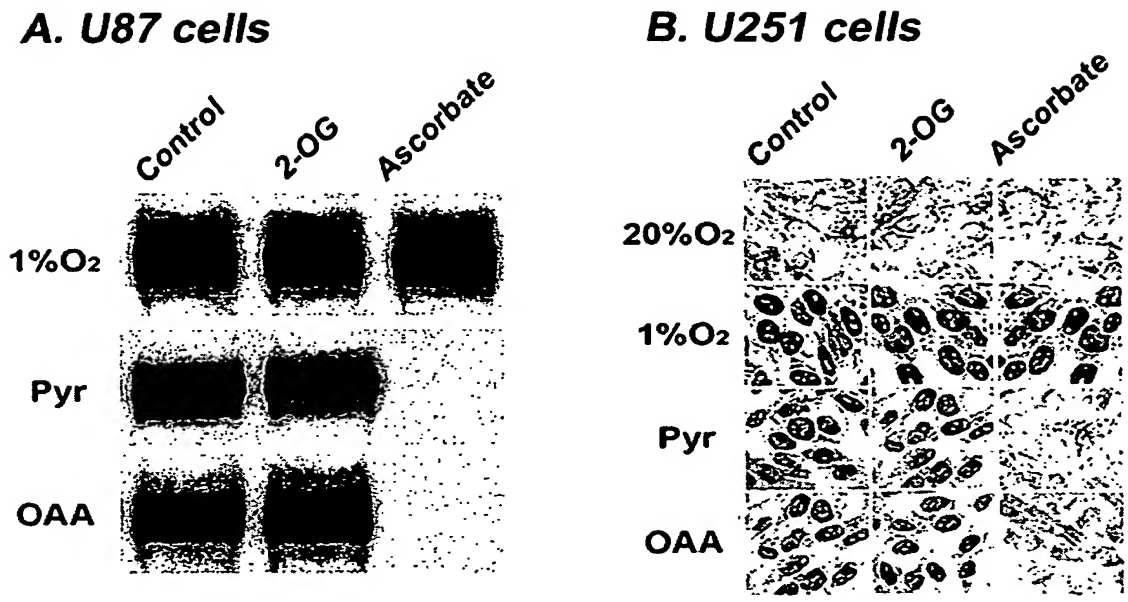

FIG. 15
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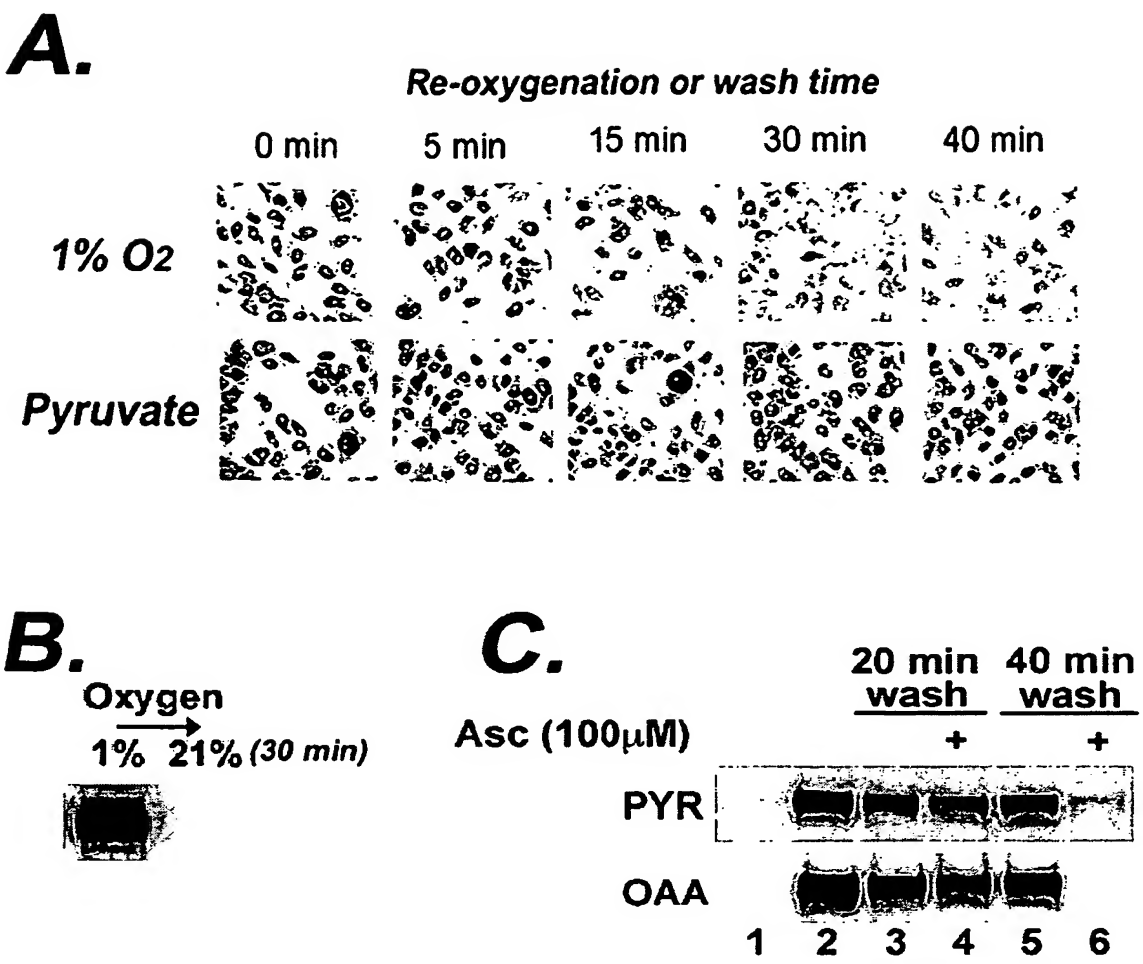

FIG. 16
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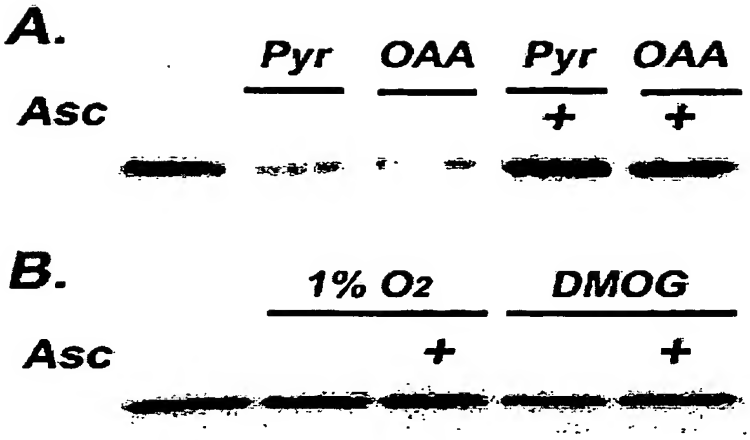

FIG. 17
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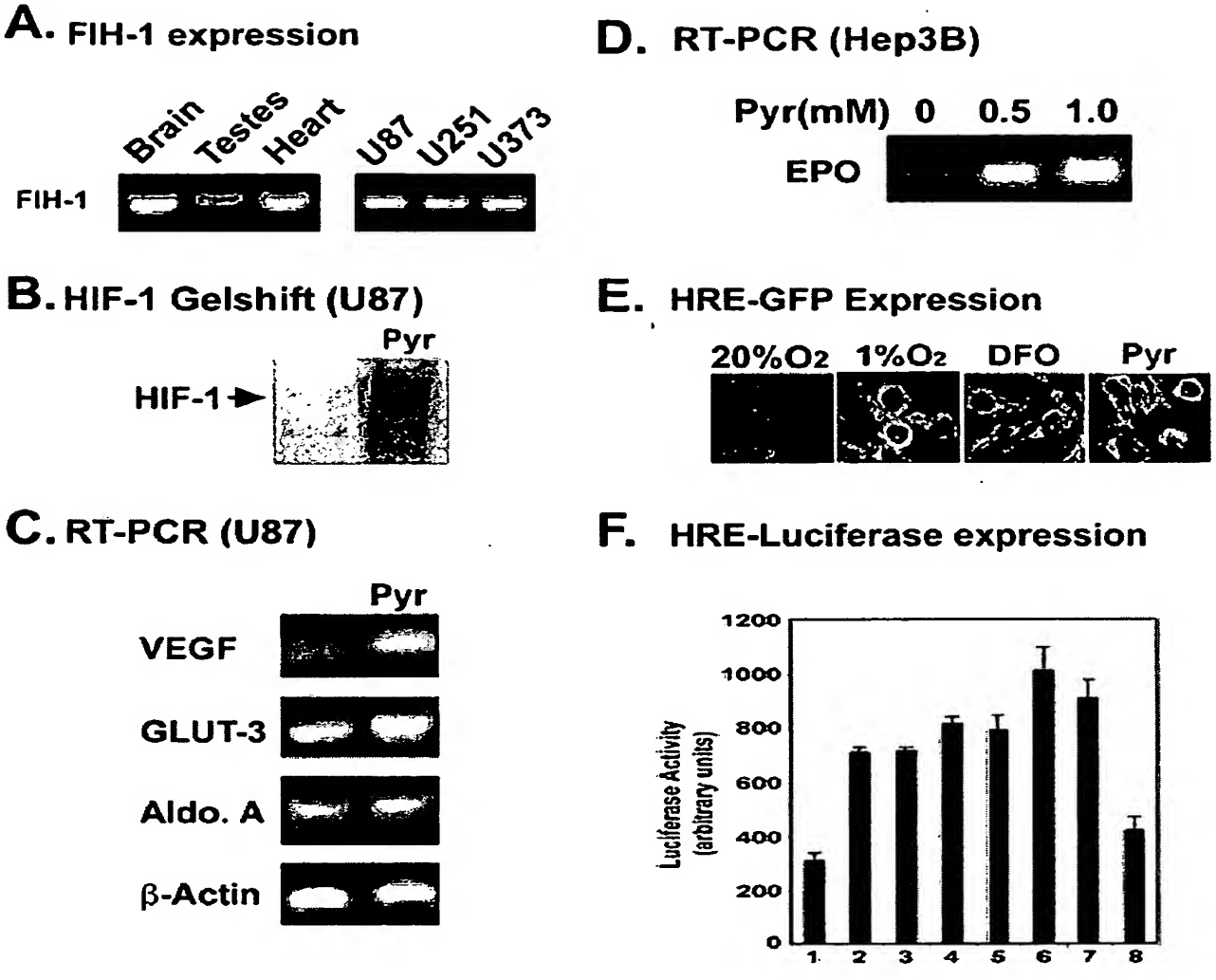

FIG. 18
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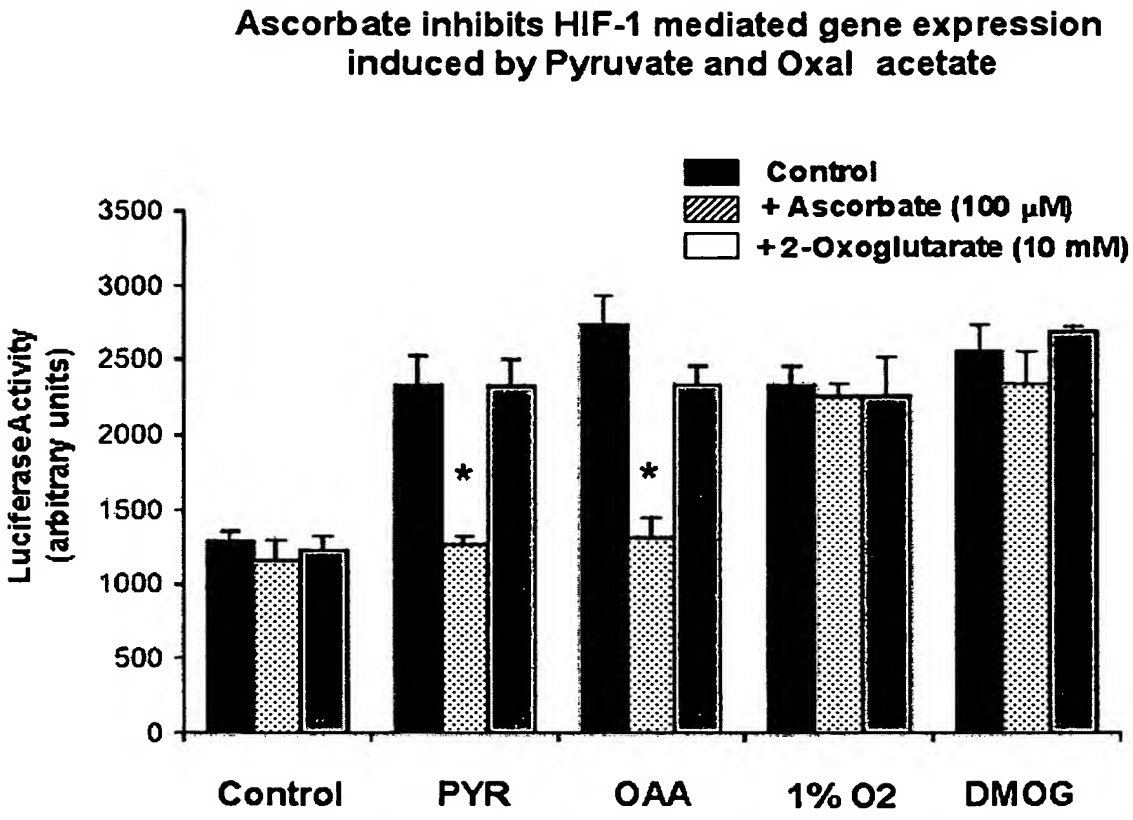

FIG. 19
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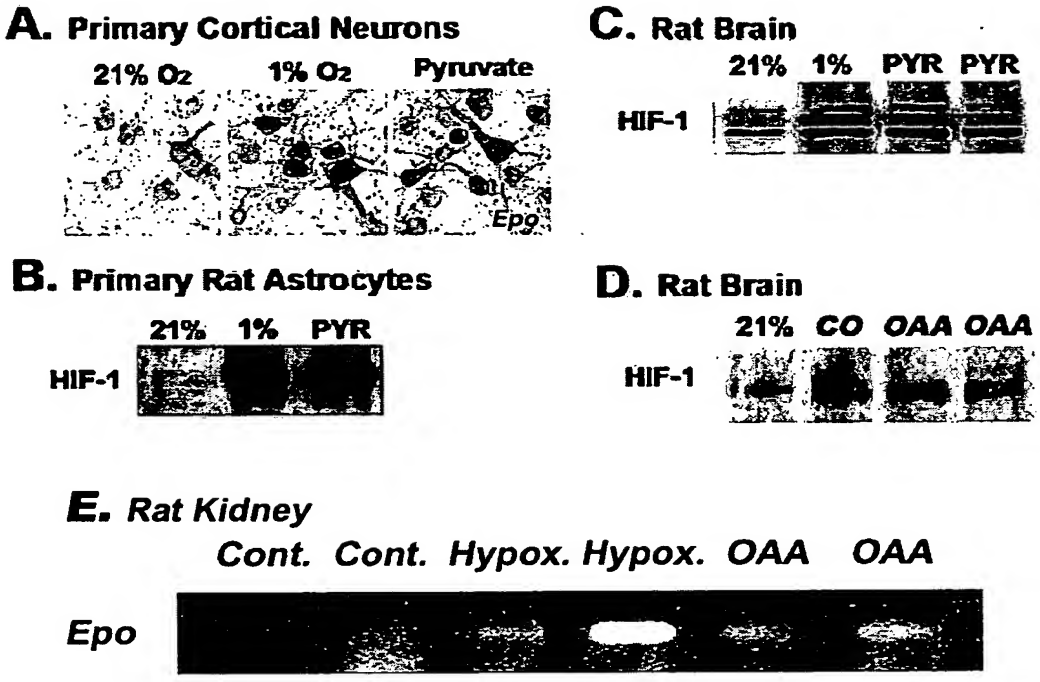

FIG. 20
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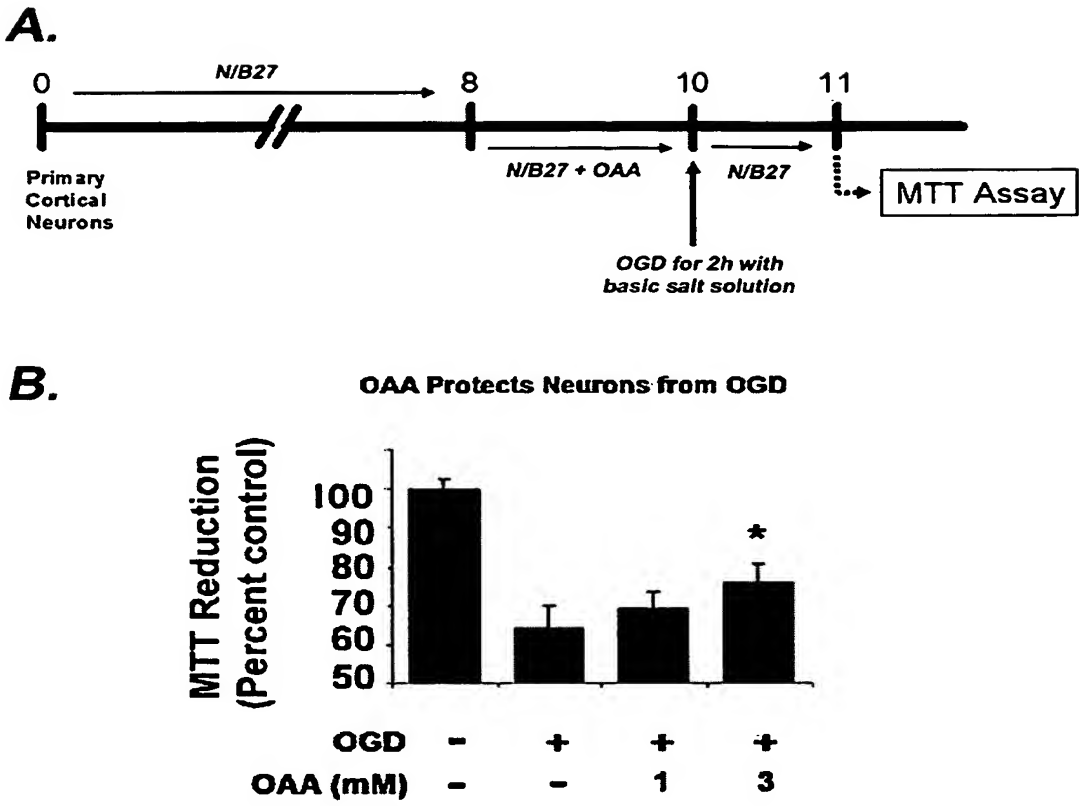

FIG. 21
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